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Gonadal Influences on Erythrocyte Reducing Capacity in the Adult Male. (26217)

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Proper function of the normal mammalian erythrocyte is largely dependent on intracellular concentration of reduced glutathione, which permits metabolic reduction reactions to proceed at a normal rate. Because of its low redox potential, reduced glutathione is easily oxidized(1,2), thereby acting as a potent reducing substance. Consequently, any diminution in capacity of the erythrocyte to

perform its usual reduction reactions suggests a defect, or deficiency, in reduced glutathione content.

The reducing capacity of the human red blood cell *in vivo* has been determined by Schwartz, Landau, and Soffer by measuring the extent to which an intravenous infusion of sodium thiosulfate altered ability of the erythrocyte to reduce iodine to iodide(3). In a group of normal adults of both sexes, sodium thiosulfate administration consistently

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produced a greater increase in erythrocyte reducing capacity in the adult female than in the adult male. The results were expressed in terms of a "reductivity index," which bears an inverse relationship to changes in red blood cell reducing activity, and relates these changes to amount of sodium thiosulfate infused. One castrated male was also studied, who exhibited a reductivity index within the range for normal females. Administration of testosterone to this patient, as well as to 3 normal female subjects, resulted in a significant fall in reductivity index toward normal male levels. Stilbestrol administration to 2 normal males produced a rise in reductivity index, to approach the normal female range. These phenomena suggested a relationship between gonadal secretions and erythrocyte reducing capacity(3).

The present study of a group of untreated adult male subjects with evidence of gonadal dysfunction is directed toward correlating the reductivity index with levels of urinary gonadotrophins, to evaluate further the influence of gonadal function on erythrocyte metabolism.

Materials and methods. Eight adult male subjects with disturbances in gonadal function were studied. Two patients had hypopituitary hypogonadism, one hypogonadotrophic hypogonadism, one disseminated carcinoma originating in the lung, and one subject had carcinoma of the pancreas. All 5 patients exhibited reduced quantities of urinary gonadotrophins, as measured in 24-hour specimens by the kaolin adsorption method of Bradbury *et al.*(4), as applied to the mouse bioassay technic(5). Of the remaining patients one had pancreatic carcinoma, associated with elevated gonadotrophin excretion. Another had slight bilateral testicular atrophy, without other clinical evidence of hypogonadism, but with striking excesses of urinary gonadotrophins. The third patient had clinical evidence of hypogonadism with small testes, but with normal urinary gonadotrophin levels. The ages varied from 20 to 77 years. All patients had previously undergone normal, spontaneous puberty, except for the 20-year-old male with hypogonadotrophic hypogonadism in whom pu-

berty had not yet occurred (Table I). No patient had received gonadal hormonal treatment for at least 3 years prior to observation.

Studies were performed in the fasting state, prior to and after an intravenous infusion of 100 ml of 10% sodium thiosulfate (Kirk), following the procedure outlined by Schwartz, Landau, and Soffer(3). Except for occasional nausea and one episode of mild vomiting, no untoward effects were observed in any of the patients studied. Normal values for the reductivity index in this laboratory are reported elsewhere(3) to be from 28 to 42 (with a standard deviation of 5) for normal adult males, and from 68 to 127 (with a standard deviation of 17) for normal adult females.

Results. A significant elevation of reductivity index was observed in 5 of the 8 male patients (Table I), all of whom had reduced urinary gonadotrophin titers. The highest reductivity index was 72.8, which fell within the normal female range. This occurred in the young adult patient (No. 1) with hypogonadotrophic hypogonadism, thus suggesting that prepubertal male values may be of comparable magnitude. Two patients (Nos. 6 and 7), one with slightly elevated, and the other with normal urinary gonadotrophins, showed a modest increase in reductivity index to 45.8 and 42.2, respectively, both values just exceeding the highest level previously obtained in normal male subjects. These latter 2 values were not statistically distinct from normal, and suggested that only a partial androgen deficiency may have existed. One patient (No. 8), with markedly elevated urinary gonadotrophin titers, but no clinical evidence of hypogonadism, possessed a normal reductivity index of 29.0. No correlation was observed between adult age, body size, or erythrocyte sedimentation rate and reductivity index.

Discussion. The above results suggest that a normal erythrocyte reducing capacity in the human male depends, at least in part, on presence of adequate amounts of circulating androgens. When gonadal secretion is impaired, as indicated by either insufficient or excessive urinary gonadotrophin titers, a decrease in red blood cell reducing

TABLE I. Relationship of Reductivity Index to Gonadal Function in Adult Males.

Sub- ject	Age, yr	Race	Reduc- tivity index	Urinary go- nadotrophins, muu/24 hr*	Hemato- crit, %	Erythrocyte sedimenta- tion rate, mm/hr	Clinical condition
1	20	N	72.8	Neg. at 5	44.5		Hypogonadotrophic hypogonadism Negative cell chromatin pattern Normal sella turcica
2	32	W	59.4	<i>Idem</i>	37	32	Carcinoma of pancreas Normal sella turcica
3	77	N	54.4	"	42	46	Carcinoma of lung with metastases Normal sella turcica
4	56	W	49.1	"	36	107	Chromophobe adenoma with second- ary hypogonadism and myxedema Diabetes mellitus Idiopathic hyperlipemia
5	58	W	48.6	"	48		Chromophobe adenoma with second- ary hypogonadism
6	68	W	45.8	Pos. at 50	35	45	Carcinoma of pancreas
7	32	W	42.2	Pos. at 10 Neg. at 50	43		Primary hypogonadism with azo- spermia and testicular immaturity Negative cell chromatin pattern Normal sella turcica
8	32	W	29.0	Pos. at 400	42.5	27	Slight bilateral testicular atrophy Spermiogenesis present (biopsy) No clinical hypogonadism Negative cell chromatin pattern Normal sella turcica Very high protein-bound iodine without hyperthyroidism

* Mouse uterine units/24-hr urine volume.

capacity occurs, reflected by a rise in reductivity index toward the normal female range. Since the reducing capacity of the erythrocyte probably mirrors the intracellular concentration of reduced glutathione, the presence of hypogonadism in the male is presumably related to an interference with the activity of reduced glutathione, probably by shifting the erythrocyte glutathione equilibrium in favor of the oxidized form.

Summary. The effect of an intravenous infusion of sodium thiosulfate on erythrocyte reducing capacity was determined in 8 male patients with disturbances of gonadal function. A reductivity index was then determined for each patient which is inversely proportional to the erythrocyte reducing capacity. In 5 patients with reduced urinary gonadotrophin titers, the reductivity index was significantly higher than that of normal

males. In the 3 remaining patients with normal or elevated urinary gonadotrophins, there was a normal or equivocally elevated reductivity index. These results indicate that hypogonadism in the human male may be associated with a decrease in reducing capacity of the red blood cell. This probably reflects an inadequate activity of reduced glutathione in the erythrocyte.

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Alterations in Aorta Lipids with Advancing Atherosclerosis.* (26218)

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Although it has long been possible to perform reasonable quantitative analyses of the lipids of normal and diseased arteries(1-4) only recently have such analyses become rapid, convenient, and accurate on a very small scale. This has been largely due to two advances in technic: silicic acid chromatography, which permits separation and analysis of relatively small samples of lipids into several classes; and gas-liquid chromatography, which permits separation and analysis of fatty acids derived from such lipids on a very small scale. These technics, permitting complete analyses on many more samples than was previously possible, should facilitate testing of the several hypotheses concerning the origin of the atherosclerotic lipids. For example, are lipids which accumulate in the arterial wall in this disease formed *in situ*(5) or do they result from deposition of a portion of the blood lipids(4,6); and, in either case, is their resistance to removal due to the saturated or other high melting properties of their fatty acids(7) or to the nature of the lipids in which they find themselves?

There have been a few attempts to apply these modern technics to these problems(8-11). In general, however, these have been preliminary reports on only a few subjects, and not concerned with alterations in the atherosclerotic lipids with progress of the disease. However, Böttcher and his co-workers(10) analyzed both lipids and fatty acids in 4 stages of atherosclerosis in aortas and also, in some cases, in coronary arteries and the circle of Willis.

Our experiments have been carried on for several years with cooperation of the Pathology Dept. of this University.[†] The aim

has been to perform as complete an analysis as possible of lipids involved in development of the atherosclerotic deposits with the hope that such detailed information might serve as a basis for theories of the mechanism of lipid accumulation and, ultimately, in formulating measures for its prevention.

Four main lipid fractions (sterol esters, triglycerides, sterols and phospholipids) have been separated by silicic acid chromatography, and have been equated to the extent of the disease in the aortas from which they were extracted. Fatty acids of the sterol esters have been analyzed by gas-liquid chromatography.

Methods. Preparation of tissues. Aortas were graded from 0-4 depending on extent and severity of plaques at time of autopsy and were stored at -20°C until use.[‡] After thawing, the adventitia was stripped from the media and intima and was discarded. No attempt was made to separate media and intima because of the impossibility of such a separation in advanced stages and the desire to maintain comparable conditions. For similar reasons, whole tissues were analyzed rather than plaques alone (By far the greatest part of the lipid was present in the plaques of the diseased aortas). The tissues were cut into small pieces, lyophilized and dried under high vacuum.

Extraction of tissues. The dried tissues

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‡ (See footnote †). Only fresh samples, taken from autopsies performed at Univ. of California Medical Center, Los Angeles, were used. Grading was performed on basis severity and extent of sub-intimal fibrosis and lipid deposition and, in advanced cases, calcification of plaques. Subjects were patients at the Medical Center before death. Average ages were: for grade 0, 32; grade 1, 61; grade 2, 64; grade 3, 67 and grade 4, 71 years. Although it is difficult to find adult grade 0 aortas, we were fortunate in this respect. One patient, a chronic alcoholic with liver cirrhosis, was 52. All patients with plaques of grade 2 or greater had moderate to severe arteriosclerotic symptoms.

were extracted in the Waring blender with methylal-methanol (4:1) using approximately 60 ml of solvent per 5 g of tissue for the first extraction followed by 5 subsequent extractions with 30 ml. The solutions were clarified by centrifugation and evaporated almost to dryness at 40°C under a stream of nitrogen. Final drying was accomplished under reduced pressure. The lipid was generally a clear orange tacky grease unless the presence of considerable free cholesterol resulted in formation of crystals. The samples were dissolved in pentane, separated by centrifugation from any small amounts of insoluble material, and stored at -20°C until use.

Chromatography. All chromatograms were carried out in duplicate except for 2 grade 0 samples for which too little lipid was obtained. The method used was a modification of the system of Fillerup and Mead(12). Silicic acid (Mallinckrodt 100 mesh) was washed 7 times with acetone, allowing the denser particles to settle for a few minutes before decanting the supernatant suspension. A slurry of this silicic acid in acetone was poured into a 19 mm diam. column to a height of 95 mm. It was then washed with successive 100 ml portions of methanol, ethyl ether and pentane. The sample was transferred to the column with small volumes of pentane. Twenty ml fractions (1 col. vol.) were then collected using the following solvent mixtures and collecting 5 fractions for the first solvent and 10 thereafter: pentane, 2%, 5%, 20% ether in pentane, ether and methanol.[§] The solvent was removed from the fractions in a warm water bath under a stream of nitrogen and each fraction was dried at reduced pressure and weighed. Estimation of per cent of each lipid was accomplished with the aid of a bar graph (Fig. 1) and with careful examination of intermediate fractions. Slight overlapping of the triglyceride with the sterol and



FIG. 1. Chromatographic separation of duplicate samples of aorta lipid. Abscissa: Small numbers designate column volumes of solvent (C.V.). Solvents (S.) are: pentane (P.), % ether in pentane (large numbers) and methanol (M.). Lipid fractions (F.) are: hydrocarbons (HC.), sterol esters (S.E.), triglycerides (T.G.), sterols (S.), partial glycerides (G.) and phospholipids (P.L.).

the sterol with the partial glyceride fraction was noted. Average recovery from the columns was 94%.

Sterol ester fractions were transmethyated using 1% methanolic sulfuric acid at 70°C for analysis of their component fatty acids by gas-liquid chromatography.||

Results. In Table I are shown percentages of the several lipid fractions in the aortas of different stages. Salient points are: the proportion of triglyceride is low and does not change significantly with progress of the disease; sterol esters increase rapidly in the lower stages, then remain constant; free sterols increase irregularly throughout plaque development; phospholipids decrease rapidly from the 0 to 1 stages, then more slowly; ratio of total sterol to phospholipid is probably the best index of extent of the disease.

In Table II are shown the percentages of the major identifiable fatty acids of the sterol esters of the aortas of different stages. No significant changes are apparent with increased severity of atheromatosis. Linoleic acid appears to be the major fatty acid followed by oleic and palmitic.

[§] Although the phospholipid fraction can be readily separated into subfractions, this was not attempted because of the added great complications of fatty acid analysis which would be required. A preliminary report of such an analysis has appeared in the literature(13).

|| Gas-liquid chromatographic analyses were performed with the Wheelco Model 10 apparatus (Barber-Colman Co.) with a 6 ft. 6 mm I.D. column of ethylene glycol-succinate polyester on 60-100 mesh celite at 175-180°.

TABLE I. Composition of Lipids from Intima and Media of Aortas with Different Degrees of Atherosclerosis.

Grade	No. of subjects	Lipid, % of dry wt	% of total lipid $\pm$ stand. dev. where possible					Ratio: Total sterol/phospholipid
			Hydro-carbon	Tri-glyceride	Di- & mono-glyceride [†]	Sterol ester	Free sterol	
0	5	6.5 $\pm$ 2.0	3.5	7.2 $\pm$ 1.8	1.5	15.5 $\pm$ 6.6	18.8 $\pm$ 2.6	34.3 $\pm$ 8.5
1	3	16	1.0	9.3	1.2	35.2	23.4	58.6
2	4	10.5 $\pm$ 4.1	1.6	7.1 $\pm$ 2.1	.7	41.5 $\pm$ 2.9	21.5 $\pm$ 2.4	63.0 $\pm$ 4.0
3	2	16	.8	8.6	1.0	42.4	25.1	67.5
4	5	22.6 $\pm$ 3.1	1.0	6.3 $\pm$ 1.8	.8	41.5 $\pm$ 6.2	30.2 $\pm$ 6.3	71.7 $\pm$ 2.8
								48.3 $\pm$ 10.7
								22.6
								20.1 $\pm$ 5.7
								15.7
								15.2
								.8
								2.6
								3.4
								4.3
								4.8

Discussion. The results shown above indicate clearly that with progress of atheromatous disease, the nature of the deposited lipid undergoes a steady change in the direction of an increased proportion of total sterol and a decreased proportion of phospholipid. This is in general agreement with the results of Böttcher and his co-workers(10) although the absolute values are quite different. It is not clear whether this is the result of a long continued exposure to blood lipids in which the sterol/phospholipid ratio is too high, although this has been considered an attractive hypothesis(4,6).

The picture presented by these lipid changes is one of increasing metabolic inertness, and the types of lipids predominating in the later stages are typical of tissues with a very slow turnover rate regardless of their component fatty acids. This observation is also borne out by the finding of high peroxide values in lipids of advanced plaques.[†] It is difficult to see how such peroxides could accumulate if there were any reasonable turnover of fatty acids. This inertness is not inconsistent with the morphological changes occurring in the progressively necrotic plaques but may also be due to other causes. The origin of the lipid deposits, whether from blood lipids or by synthesis within the tissue, is not explained by this study, although some light may be shed by the fatty acid composition of the lipids. Nevertheless, it appears that, regardless of the route by which the lipids reach the intima of the arteries, consideration should be given to the enzyme pattern of a tissue which permits them to remain and accumulate.

The constancy of the fatty acid composition of the sterol esters with progressive atheromatous disease does not agree with the findings of Böttcher and co-workers(10) who reported an increase in the linoleate-saturated acid ratio nor does it support the hypothesis of Sinclair(7) that accumulation of lipid may be related to inclusion of saturated or other high-melting fatty acids. Further

[†] The high peroxide numbers of lipids of advanced atheromatous plaques(14) have been confirmed in this laboratory.

TABLE II. Fatty Acid Composition of Cholesterol Esters from Aortas with Different Degrees of Atherosclerosis.

Grade	No. of samples	% of major fatty acids					Arachidonic
		Palmitic	Palmitoleic	Oleic	Linoleic	Eicosatrienoic?	
0	4	12.4	3.3	30.6	41.2	3.0	6.5
1	3	13.4	4.8	29.2	42.7	1.1	6.8
2	4	10.6	3.4	27.2	47.6	2.4	7.5
3	2	12.6	3.2	26.3	49.3	1.0	5.6
4	4	13.1	5.6	27.4	42.1	1.6	7.3

study should reveal whether these differences may be explained by changes in diet.

Summary. Analysis of lipids of the intima and media of aortas with progressive stages of atherosclerosis reveals an increase in sterol and sterol ester fractions and a decrease in phospholipids. It is proposed that these changes reveal a very low rate of lipid turnover in this tissue. The fatty acid composition of the cholesterol esters did not change with advancing atherosclerosis.

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A Ventilatory Effect of Carbonic Anhydrase Inhibition in Man. (26219)

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An increase in resting ventilation has been a consistent finding in dogs given the carbonic anhydrase inhibitor acetazolamide(1, 2,3). In man, however, no increase in resting ventilation has been reported even though doses of the drug were well within the range which produced a marked effect in dogs(4,5, 6). In seeking a reason for the absence of an effect in man in the reports, it was noticed that the drug was usually given intravenously

into the dogs but invariably was taken orally by the human subjects. If insufficient time during the experiment was allowed for absorption and attainment of a sufficient concentration of drug in the blood, the ventilatory response of man to carbonic anhydrase inhibition might well not have been seen. In the experiments described below, the results obtained in man with the same dose of drug given orally and intravenously are compared.

Methods. The subjects were 5 normal males ranging in age from 21 to 36 years. To collect expired gas, the subject wore either a rubber gas mask with a hose adapter on the expired valve port or a mouthpiece and valve arrangement and a nose clip. The expired gas was led through a mixing cylinder of 1.5 liters and from there through a dry test gas meter. The mixed expired gas sample was taken through a 13 gauge needle inserted into the rubber tubing at a point adjacent to the distal opening of the mixing chamber. The sample gas was pulled serially at a known rate through a drying tube, a Beckman Model E3 oxygen analyzer, and a Liston-Becker Model 16 CO₂ analyzer. From readings of the dry test gas meter and the gas analyzers, the minute volume of ventilation, CO₂ output and oxygen uptake were calculated. Frequency of respiration was also recorded.

Experiments were always run in the morning. The subject rested in the supine position on a cot for approximately 30 minutes prior to beginning the experiment. The gas mask or valve and nose clip were adjusted during this period and the subject was allowed at least 10 minutes to become accustomed to it. Control measurements were made for 30 minutes after beginning the experiment. The dry test meter was read every 6 minutes during which gas analyzers were read every 2 minutes and the 3 readings were averaged for the 6-minute period. Frequency of respiration was recorded during second and fifth minutes and averaged.

At the end of control period, the subject either ingested acetazolamide in tablet form or the sodium salt of the drug was infused in saline into an antecubital vein. In both cases, subject received 25 mg/kg of body weight. For the infusion, the requisite amount of drug was dissolved in approximately 250 cc of physiological saline and was allowed to drip into the vein over a period of 12 minutes. Every subject received the drug by both routes in 2 separate experiments. In the experiments in which drug was administered orally, data were collected for 3 hours after ingestion. When the drug was given intravenously, experimental period was contin-

ued for 2 hours after beginning of infusion.

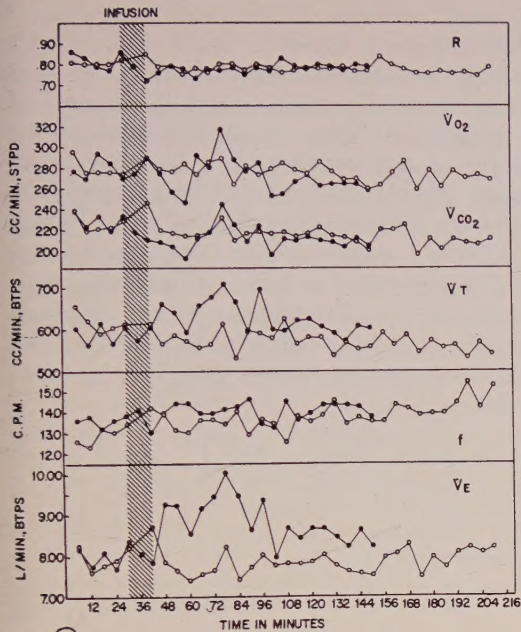
Two of the subjects, in a third experiment, were given 50 mg/kg of acetazolamide intravenously. The same procedure as described for the lower dose was followed.

Results. Mean values of O₂ consumption, CO₂ output, and respiratory exchange ratio of the 5 subjects are plotted against time in experiment in Fig. 1. There appeared to be no consistent difference in metabolic gas exchange as a result of different routes of administration of acetazolamide.

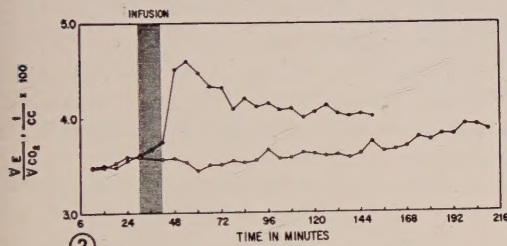
Ventilation at the bottom of Fig. 1, increased approximately 15% at the end of intravenous infusion of acetazolamide. Approximately one hour later, ventilation had returned almost to control level although it was still slightly elevated. No such increase was seen with the oral dose. Mean values of tidal volumes, also in Fig. 1 indicated that the increased ventilation after intravenous injection of acetazolamide was primarily due to an increased tidal volume.

As a more sensitive reflection of ventilatory status, ratio of ventilation to CO₂ output (the ventilatory equivalent for CO₂) was calculated and mean values for the 5 subjects are shown in Fig. 2. Approximately 2 hours after ingestion of drug, this ratio began to increase and showed evidence of a continuing increase 3 hours after ingestion. Means of the 5 determinations for each subject during control period and the last 30 minutes of experimental period were compared in a paired t-test by taking the difference for each subject. The increase in the ventilatory equivalent for CO₂ during the last 30 minutes was highly significant ($P < .01$). The venous route of drug administration produced even more pronounced effects on the ventilatory equivalent for CO₂. Comparing the period of maximum effect, the first 30 minutes after end of infusion, with the control period, the ratio was increased over 25%. After reaching a maximum, the ratio declined and approached that obtained with the oral drug, in both cases greater than during the control.

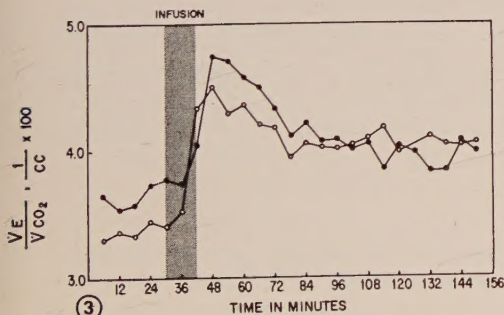
In 2 subjects given 50 mg/kg of acetazolamide intravenously, the results did not differ notably from those of the lower dose given intravenously. Mean values of the ventilatory



①



②



③

FIG. 1. Mean values of respiratory exchange ratio (R), oxygen uptake ($\dot{V}O_2$), CO_2 output ($\dot{V}CO_2$), tidal volume ($\dot{V}T_{BTPS}$), respiratory frequency (f), and expired minute volume of ventilation ($\dot{V}E_{BTPS}$) of 5 male subjects who were given 25 mg/kg of acetazolamide orally ($\bigcirc$) and intrav. ($\bullet$).

FIG. 2. Mean values of ventilatory equivalent for CO_2 ($\dot{V}E/\dot{V}CO_2$) of 5 subjects. $\bigcirc$, oral dose of 25 mg/kg; $\bullet$, intrav. dose of 25 mg/kg.

FIG. 3. Mean values of ventilatory equivalent for CO_2 of 2 subjects who were given 25 mg/kg of acetazolamide intrav. ($\bullet$) and 50 mg/kg intrav. ($\bigcirc$).

equivalent for CO_2 for the 2 subjects with the 2 doses are shown in Fig. 3 and serve to illustrate this point. Similar correspondence was seen in comparisons of the other variables.

Discussion. In dogs, inhibition of carbonic anhydrase caused a transient reduction in CO_2 output and an increase in ventilation (1), or only an increase in ventilation with no change in CO_2 output (3). For the purpose of this discussion, therefore, any significant increase in ratio of ventilation to CO_2 output (the ventilatory equivalent for CO_2), was considered to be a ventilatory effect of carbonic anhydrase inhibition.

Acetazolamide given intravenously produced a marked increase in ventilation and the ventilatory equivalent for CO_2 . The same dose taken by mouth, however, did not have an equal effect on ventilation and the effect on the ventilatory equivalent was much delayed and smaller in magnitude (Fig. 2). The delay of approximately 2 hours was probably related to time required for absorption of the drug into the bloodstream and build-up of a suitable concentration there. Two hours corresponds fairly well with the data of Maren and Robinson (7) who obtained peak plasma concentrations of drug at that time in patients given 75-85 mg/kg by stomach tube.

The increase in ventilation caused by intravenous administration of acetazolamide was more in keeping with results obtained from dogs. The magnitude of the increase, however, was much less. Both anesthetized and non-anesthetized dogs increased ventilation by approximately 100% (1,2,3). Maximum increase was only 15% in these experiments on man. The fact that doubling the dose in 2 subjects did not produce a greater effect tended to rule out the possibility that carbonic anhydrase had not been completely inhibited. If inhibition was complete, then the results in man suggest a lesser role of the enzyme in man compared to the dog.

It has been shown that in the dog, bicarbonate transport of CO_2 was reduced from 58% to 17% of total CO_2 exchanged at the lung after inhibition of carbonic anhydrase (8). This necessitated that a greater proportion

of CO₂ must be transported in carbamino combinations and in physical solution to maintain a steady state. For this to take place, the gradient for CO₂ from the tissues to alveolar air must have been increased, particularly in the case of CO₂ in physical solution. To accommodate for the inhibition of carbonic anhydrase, the subjects in these experiments neither increased their ventilation as much nor retained as much CO₂ as did anesthetized dogs(1). This implied that alveolar PCO₂ was not lowered to the same extent nor was tissue PCO₂ raised to the same extent. Since carbamino transport of CO₂ is less subject to changes in PCO₂ than the transport of CO₂ in physical solution(9), a possible explanation for the different responses of man and the dog to carbonic anhydrase inhibition was that a greater proportion of CO₂ exchanged at the lung was derived from CO₂ in carbamino combinations. It must be stressed, however, that this is purely speculative.

Aside from raising an interesting question about CO₂ transport, this study has demonstrated a ventilatory effect of carbonic anhydrase inhibition in man when the inhibitor was given orally as well as intravenously. The delay in effect of an oral dose, due probably to absorption time, has been pointed out as a possible pitfall for studies in man. Whether or not a species difference exists for the relative importance of carbonic anhydrase in CO₂ transport remains to be settled.

Summary. Five normal males were given

25 mg/kg of the carbonic anhydrase inhibitor acetazolamide both orally and intravenously in separate experiments. The oral dose caused a significant increase in ratio of ventilation to CO₂ output, but only after a delay of approximately 2 hours after ingestion. The intravenous dose caused a greater increase in ratio of ventilation to CO₂ output which was evident as soon as infusion of drug was completed. It was concluded that carbonic anhydrase inhibition in man did produce a ventilatory effect which had not been demonstrated previously when the inhibitor was given orally.

The author wishes to thank Dr. Cloyd D. Green for administering the drug intravenously and for exercising medical supervision of experiments. Excellent technical assistance of Mr. R. J. Ball is also gratefully acknowledged.

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Effect of Pitressin on Cationic Exchange and Muscular Activity in the Dibenzyline Blocked Rat Gastrocnemius.* (26220)

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We have previously reported that in rats, high doses of adrenergic blocking agents diminish running ability as measured on a treadmill(1). With this loss of ability to perform

work the transmembrane cationic exchanges ordinarily mirrored in the plasma in association with muscular exercise are also proportionately reduced. Since these adrenergic blocking agents are equally effective in stopping contraction of the isolated rat diaphragm

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stimulated either directly or indirectly, it is evident that this is not due to a hemodynamic effect(2). On the contrary, it is possible that adrenergic blocking agents inhibit muscular performance directly by interfering with ionic movements across the cell membrane.

Pitressin, even in small doses, has been reported to shift water and sodium out of the extracellular space even in presence of adrenergic blockade(3), and into the cells of at least one tissue, the aorta(4). It has also been shown to be effective in partially restoring work performance in the adrenalectomized-hypophysectomized rat(5). On this basis, preliminary experiments were carried out which showed that a single dose of intravenous Pitressin could restore running ability almost completely in rats immobilized by adrenergic blockade. The present experiments were designed to study this response in detail.

Methods. Adult male albino rats of an inbred Wistar strain, 6 months of age and weighing 270-310 g, were used. The rats were anesthetized with pentobarbital (3.33 mg/100 g body weight) and phenobarbitone sodium (6 mg/100 g) administered intraperitoneally and subcutaneously respectively. The kidneys were then decapsulated and the pedicle ligated. An inulin solution, approximately 25% w/v was injected into the tail vein in a dose of 0.5 ml/100 g body weight. The absolute amount of inulin injected was accurately determined in each case by analysis of an aliquot taken from the injection syringe. After 3 hours of equilibration, polyethylene catheters were implanted into the right femoral artery and vein, an initial blood sample (0.9 ml) was taken from the artery and a control specimen excised from the right gastrocnemius muscle. Dibenzylene[†] at a dose of 250 μ g/100 g body weight in 0.2 ml of saline was introduced into the femoral vein and the animal at once prepared and mounted for kymographic recording of contractions of the left gastrocnemius activated by stimulation of the cut end of the sciatic nerve. The gastrocnemius muscle was then stimulated at a rate of 20 twitches/second for 20 minutes.

[†] Dibenzylene was supplied through the courtesy of Smith, Kline and French.

A dose of 80 mU of Pitressin in 0.2 of saline was given intravenously when the effect of Dibenzylene was close to its peak and muscular contractions had not entirely ceased. Control animals were sham operated and received comparable volumes of saline. After 20 minutes stimulation a second blood sample was taken through the femoral cannula and the left gastrocnemius muscle was quickly excised.

Intracellular water, sodium and potassium determinations were carried out, with minor modification, by the methods of Ledingham(6) as follows: muscle samples were blotted with Whatman No. 42 filter paper, cut into several pieces and weighed at once, then either dried to a constant weight at 105°C for determination of water content or used for determination of muscle inulin according to the method of Ross and Mokotoff(7). Muscle samples used for estimation of total water content were then extracted free of fat by the method of Hastings and Eichelberger(8) and also used for sodium and potassium determinations. Dried samples were wet ashed in 1.0 ml of concentrated nitric acid, diluted and analyzed by flame photometry. Results are expressed as meq/l intracellular fluid. Blood was analyzed for glucose by the method of Nelson(9) with the copper reagent as modified by Somogyi(10), and for lactic acid by the method of Barker and Summerson(11). Plasma was analyzed for inulin by the method of Higashi and Peters(12) and for sodium and potassium by flame photometry. The volume distribution of inulin was taken as a measure of extracellular water. The amount of intracellular water per kg of fat-free muscle was estimated by subtracting extracellular water/kg fresh fat-free muscle from the measured water content of the whole muscle. Intracellular sodium and potassium were calculated by deducting from the total amount of tissue ions the amount contained in a known volume of extracellular water.

Thirty-five rats were divided into 4 groups. Group 1 (7 rats) served as untreated, sham operated control; group 2 (8 rats) was given Dibenzylene (250 μ g/100 g intravenously); group 3 (8 rats) was given Pitressin (80 mU

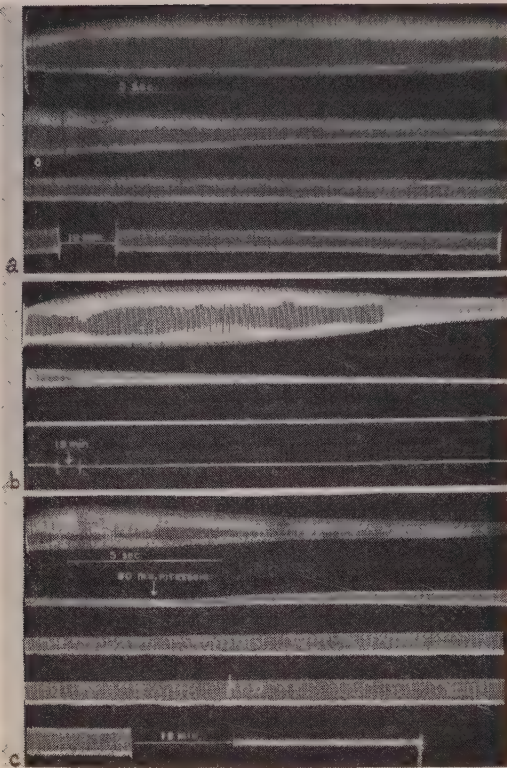


FIG. 1. Responses of adult rat gastrocnemius *in situ* to sciatic nerve stimulation, 20 twitches/sec. (5-6 V, 0.2 msec. duration) for 20 min. (a) Normal control, (b) following intrav. Dibenzyline 250 µg/100 g, (c) following intrav. Dibenzyline countered with Pitressin.

intravenously) and group 4 (12 rats) both Dibenzyline and Pitressin.

Results. Effect of Pitressin on Contractility of Dibenzyline Blocked Gastrocnemius *in situ*. When the gastrocnemius was stimulated indirectly at 20/second the height of contraction, except for an initial unstable period of less than half a minute, remained essentially unchanged throughout the 20 minutes of stimulation (Fig. 1a). In sharp contrast, the height of muscle contraction decreased markedly after Dibenzyline so that within a few minutes only a scarcely discernible wave remained to mark each stimulus (Fig. 1b). When 80 mU of Pitressin was administered intravenously before the contractions had entirely diminished, Dibenzyline suppression was eliminated for a period of 12-16 minutes before the effect wore off (Fig. 1c). These effects were entirely reproducible.

Effect of Pitressin on Ion Exchanges in Di-

benzylene Blocked Gastrocnemius *in situ*. The essential changes in intracellular and extracellular water, sodium and potassium corresponding to the changes in contractility just described are shown in Fig. 2.

In the control group, intracellular water and sodium increased while potassium decreased following the period of work as previously described(2). Dibenzyline hindered the cationic exchange as it suppressed contractility but did not much affect the water shift. Pitressin was effective in restoring the transmembrane movements of sodium and potassium in the Dibenzyline treated animal as it restored contractility. Pitressin alone did not affect the sodium, potassium or water shift produced by exercise.

Discussion. Ingle and Li reported that while an adrenal cortical extract could not entirely correct the work deficit of adrenalectomized-hypophysectomized rats such an extract given together with a very small amount of vasopressin could normalize work performance(5). We later reported that Pitressin produced a movement of sodium and water out of and potassium into the extracellular space(3). Daniel *et al.* showed this to mirror, in part, shifts into and out of cells in the aorta wall(4) and we have now broadened this to include skeletal muscle as well (in preparation). The present experiments suggest that these observations are all interdependent.

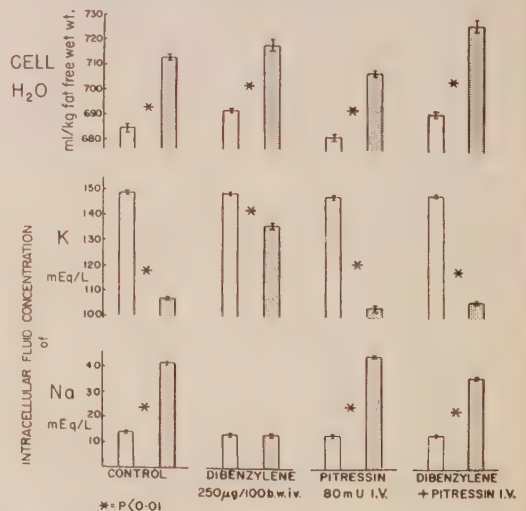


FIG. 2. Effect of stimulation of gastrocnemius *in situ* on intracel. water, Na and K in intact, Dibenzyline and Pitressin treated adult rats.

It is generally held that the action potential which triggers the contractile process depends on a transmembrane ionic exchange characterized first by an inward movement of sodium followed rapidly by an efflux of potassium (13). Adrenergic blocking agents, in relatively high doses, have been shown to interfere with the transmembrane exchange of ions in resting muscle incubated *in vitro* and this is accompanied by an impaired contractility measured *in vivo*, *in situ* or *in vitro* (2). Pitressin, which can shift sodium and potassium in the continuing presence of such an adrenergic blockade, restores contractility to the rat gastrocnemius during the limited period of its effectiveness. Perhaps the observation of Ingle and Li rests on the same ability of Pitressin to transfer sodium and potassium (with water) across the cell membrane.

Summary. Intracellular sodium concentration of stimulated rat gastrocnemius muscle increased 3-fold while potassium decreased by almost one-third after 20 minutes of stimulation (20/sec.). Dibenzylene abolished these changes and strikingly reduced the amplitude of muscular contraction. Pitressin (80 mU intravenously) in Dibenzylene treated rats restored both the cationic shifts and muscular

contractions during its period of effectiveness. We suggest that the ability of Pitressin to restore work performance is brought about by a direct action on cationic exchanges across the muscle membrane.

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Immunologic Mechanism of Transmission of Experimental Glomerulonephritis in Parabolic Rats.* (26221)

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We reported (1) that in patients with glomerulonephritis antibodies against human kidney could be detected by means of the very sensitive but also very delicate collodion technic. These findings were confirmed by Pfeiffer *et al.* (2) and by Brod (3) with the same technic and by Vorlaender (4) with a sensitive complement fixation technic. More recently Liu and McCrory (5) using the

tanned red cell technic and the Ouchterlony gel-precipitation method have come to identical conclusions. On the other hand, Heyman (6) and Goodman (7) could not find such antibodies. All investigators who demonstrated such antibodies agreed with us that antibody titers were lowest or absent in the acute stage of glomerulonephritis and became higher and more consistent later in the disease. We assumed at first that in the acute stage the antibody was completely absorbed by the kidney which was supposed to act like

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TABLE I. Parabiosis of Nephritic Rats.

No. of animals	Primary member	Secondary member
	Duration of disease on day of parabiosis (days)	Duration of parabiosis on day of sacrifice (days)
7	8	14
3	16	10
3	16	14
5	45	14
2	55	8

a sponge leaving no or very little free antibody in the circulation. This assumption, however, is not tenable. For we could show (8) that in the experimental rabbit disease produced by antirabbit kidney duck serum, the serum of an animal with acute glomerulonephritis on the second or third day of the disease can produce immediate nephritis when transfused to a sensitized but normal rabbit. This corresponds to a passive transfer of antibody. Therefore, at least in this experimental disease antibody is present in excess practically from onset and is not completely absorbed by the kidney.

One may thus be forced to assume that the antibody against healthy kidney tissue found in human glomerulonephritis and occurring rather late in the disease is possibly not the original antibody causing the disease. It may rather be the result of destruction or alteration of kidney tissue, which then acts as an antigen giving rise to this secondary antibody of unknown significance. To clarify the role of such an antibody which appears late in the disease, we followed the suggestion of Pfeiffer(9) and performed the following experiments.

Methods. Female rats of the Sprague-Dawley strain, weighing approximately 150 g, were made nephritic by intravenous injection of 0.8 ml of antirabbit kidney serum. They showed a continuous 4+ proteinuria which began within 24 hours. A series of them was sacrificed at daily intervals during the first 10 days of disease. Others, as shown in Table I were then parabiosed to normal rats according to the method of Bunster and Meyer, by lateral attachment of the peritoneum, but without creating a common peritoneal cavity. Twenty parabioses of such

pairs were successfully performed. By intravenous injection of fluorescein into the tail vein of one partner and appearance of a fluorescent urine in the other partner, one can find that it takes approximately 5 days before a good vascular connection between the two partners is established. Following parabiosis, attempts were made to collect daily urines of primary and secondary partners. This was not always possible, since such urines had to be expressed manually from the bladder to avoid mixture with the urine of the other partner. Thus only percentage of protein in the urine could be measured but no 24 hour outputs. In general, after the 5th day of parabiosis, the proteinuria in the secondary partner increased from the value, maximally 100 mg %, found in normal rats to 200 to 800 mg %.

Results. Table I shows duration of disease of the primary nephritic partner in the different groups when he was parabiosed to a healthy partner, and duration of parabiosis before sacrifice. Table II shows a typical example of the proteinuria in a parabiosed pair.

When 6 healthy pairs of rats were parabiosed, no abnormal proteinuria was observed except in some on the day of operation.

Both groups of parabiosed animals and the single animals of the first to the 10th day of disease were then sacrificed and their kidneys studied by routine histology and by Coon's technic of fluorescein labelled antibodies, but using fluorescein isothiocyanate as conjugating agent rather than fluorescein isocyanate. All kidneys of the single animals and of the primary partners showed an intense staining

TABLE II. Proteinuria in Parabiotic Rats (mg %).

Day	Primary partner	Secondary partner
-4	560	90
-2	980	70
-1	540	105
	Parabiosis	
+ 2	1290	116
+ 3	560	anuric
+ 6	1500	380
+ 7	1000	400
+ 8	700	anuric
+ 9	1200	800
+12	1100	760

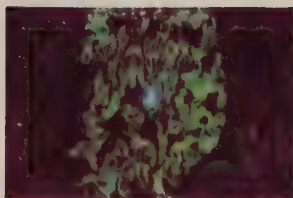


FIG. 1. Cryostat section ($3\ \mu$) of kidney of rat 1 day after 0.8 ml antiratkidney rabbit serum i.v. Stained with antirabbit gamma globulin horse serum conjugated with fluorescein isothiocyanate.

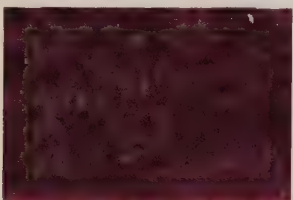


FIG. 2. Cryostat section ($3\ \mu$) of kidney of rat 1 day after 0.8 ml antiratkidney rabbit serum i.v. Stained with antirat gamma globulin rabbit serum conjugated with fluorescein isothiocyanate.



FIG. 3. Same as Fig. 2, but 3 days after 0.8 ml antiratkidney rabbit serum i.v.

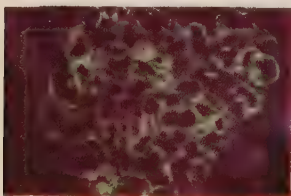


FIG. 4. Same as Fig. 2 and 3, but 7 days after 0.8 ml antiratkidney rabbit serum i.v.

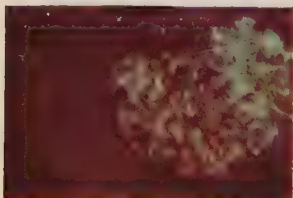


FIG. 5. Same as Fig. 2, 3 and 4, but 30 days after 0.8 ml antiratkidney rabbit serum i.v.



FIG. 6. Cryostat section ($3\ \mu$) of kidney of healthy rat parabiosed to nephritic rat for 14 days prior to sacrifice. Stained with antirabbit gamma globulin horse serum conjugated with fluorescein isothiocyanate.

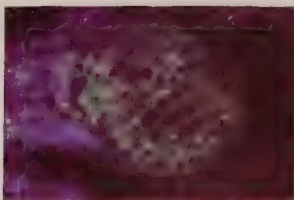


FIG. 7. Same as Fig. 6, but stained with antirat gamma globulin rabbit serum conjugated with fluorescein isothiocyanate.

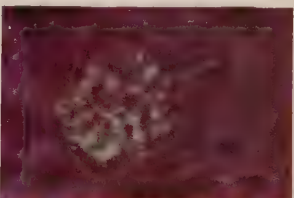


FIG. 8. Same as Fig. 7, but 32 days after parabiosis.

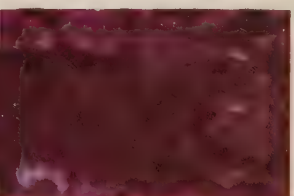


FIG. 9. Same as Fig. 7, but obtained from the parabiosis of two healthy rats.

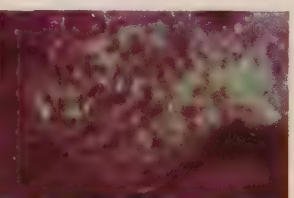


FIG. 10. Same as Fig. 7, but obtained from a rat which 5 days prior to parabiosis was unilaterally nephrectomized and then parabiosed to a nephritic rat.

with antibodies for rabbit gamma globulin from the first day (Fig. 1). The basement membrane of all glomeruli showed this intense fluorescence while all other parts of the kidney remained unstained. When such primary nephritic animals were sacrificed at daily intervals after injection of the nephrotoxic serum no staining for rat gamma globulin which would possibly indicate an auto-antibody could be obtained until the 5th or 6th day (Fig. 2, 3). Then however, the rats began to show increasing evidence for the presence of rat gamma globulin, presumably antibody, on their glomeruli. This rat gamma globulin could be demonstrated on the basement membrane for as long as 80 days after onset of the disease (Fig. 4, 5). The rat gamma globulin was indeed immunologically precipitated on the basement membrane and not encountered there in passage during the filtration process, since rat gamma globulin could not be demonstrated before the 5th to 6th day in spite of the severe proteinuria which existed from the first day. In addition, there was no staining of the glomeruli with labelled sera for rat albumin in this or any other stage, indicating that proteins which are just filtered do not stick to the glomerular membrane. Positive staining for albumin could be seen, however, in the protein droplets in the tubuli and in casts in the tubular lumina.

The secondary partners showed no staining for rabbit gamma globulin at any time (Fig. 6) indicating that at time of parabiosis no rabbit serum in demonstrable quantities could be left in the circulation of the primary partner, which could then attack the kidneys of the secondary partner. Similarly, Pfeiffer, *et al.* (9a) have reported that they too have been unable to discover any rabbit globulin on the glomeruli of the secondary partners using fluorescein isothiocyanate-labelled sera against rabbit globulin. All these kidneys showed, however, varying intensities of staining for rat gamma globulin presumably indicating the presence of an antirat kidney antibody. Degree of staining varied and not always did all glomerular loops seem to be involved. The reaction was weaker when primary partners had the disease for a long

time or only to a mild degree. No other component of the kidney stained (Fig. 7 and 8). There was no staining with labelled antisera for rat albumin or for human gamma globulin. These antisera were used as controls to demonstrate the specificity of the staining for rat gamma globulin. No partner of a parabiosis of 2 healthy rats showed any staining of the glomeruli with antisera for rat gamma globulin, or for that matter, for any other precipitation of proteins in the kidney (Fig. 9).

To confirm the specificity of the staining for rat gamma globulin further, secondary partners of 3 pairs of rats were unilaterally nephrectomized 5 days prior to parabiosis. In these animals, staining of the remaining kidney for rat gamma globulin after parabiosis was considerably more intense than in the animals with 2 kidneys (Fig. 10). Apparently, all available antikidney antibody was now concentrated on one kidney instead of 2, thus leading to an increased deposition of antibody and increased renal damage.

Microscopic examination of the kidneys of the primary partners showed the typical changes described by many investigators in experimental rat glomerulonephritis. In the secondary partner there were seen alterations in both glomeruli and tubules. The changes were more marked in the unilaterally nephrectomized animals. The glomeruli showed a varying, moderate degree of hypercellularity, and thickening and reduplication of basement membranes. No thrombi were seen in capillary lumina. Proximal convoluted tubules showed PAS positive droplets varying in size from 1 to 3 μ . These are apparently protein absorption droplets. Only few protein casts were noted. There was focal atrophy of proximal convoluted tubules.

Discussion. One may thus assume that in the experimental glomerulo-nephritis of the rat a secondary auto-antibody is formed by the glomerulo-nephritic rat which is able to attack and alter healthy glomeruli. This appears to be another instance where diseased tissue can act as an antigen which produces an antibody which in turn can attack normal tissue. One should also consider the possibility that in the disease of the primary part-

ner, an antigen-antibody complex is formed which circulates in the parabiotic pair and becomes a new antigen to which antibodies are formed. This secondary antigen-antibody complex could then cause the disease in the secondary partner similarly to the glomerulitis caused by injected beef gamma globulin(10).

It is possible that a similar mechanism plays a role in causation of subacute or chronic glomerulonephritis. Hume and Merrill(11) have reported several instances in which acute glomerulonephritis occurred in a healthy kidney transplanted from an identical twin to a patient with far advanced glomerulonephritis. Much more study will be needed to evaluate the significance of the secondary antibody in the genesis of chronic glomerulonephritis.

Summary. The glomerular basement membranes in kidneys of rats made nephritic by antirat-kidney rabbit serum show immediately the presence of rabbit gamma globulin when examined with a fluorescein labelled antirabbit gamma globulin serum. Rat gamma globulin, presumably rat antibody, appears only after 6 days on these membranes. The parabiosis of such nephritic rats to healthy rats leads to a mild but clear cut nephritis in the healthy partner with proteinuria and typical histologic changes. Rat gamma globulin, presumably antibody, but not rabbit gamma globulin can be shown on

the glomeruli of such secondarily diseased animals. The parabiosis of 2 normal rats does not lead to such a deposition of antibody or to clinical disease. Thus the primary nephritic partner seems to form an auto-antibody against its own altered kidney tissue. This antibody is then in turn able to attack the healthy kidney tissue of the secondary partner.

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Hepatic Alterations in Rats Fed 1,2-dihydro-2,2,4-trimethyl-quinoline, *Flectol H*. (26222)

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A variety of agents is capable of causing hepatic injury with attendant fatty metamorphosis, loss of cytoplasmic basophilia, and hepatic necrosis. Severe acute or chronic injury may result in fibrosis, cirrhosis, parenchymal and biliary-duct hyperplasias, and tumor formation. Relationships among these

phenomena are complex and variable. Thus, signs of injury may be reversible or progressive, while tumors may or may not be preceded by fibrosis and cirrhosis. Exploration of apparently unrelated pathways by which diverse agents produce similar ends may disclose common basic mechanisms. This report calls attention to another means for pro-

TABLE I. Part A. Liver Weight-Body Weight Ratio.

Day	Weanlings (range)	Adults (range)
1	.52-.54	.38-.45
2	.58-.65	.43-.46
3	.59-.74	.45-.52
4	.61-.73	.45-.48
5	.81-.85	.53-.60
6	.81-.98	.55-.70

ducing many of the above-mentioned hepatic changes.

During toxicological studies on a polymer of 1,2, - dihydro - 2,2,4, - trimethyl - quino-line, *Flectol H*, we noticed histologic changes limited to the livers of test rats. These included marked fatty change frequently limited to, or most prominent in, the midzonal area of the hepatic lobule, cellular alterations, pigmentation, and biliary-duct hyperplasia.

Materials and methods. *Flectol H*, a polymer of 1,2, - dihydro - 2,2,4 - trimethyl - quino-line, is an industrial antioxidant listed in the 29th edition of Monsanto's Catalogue of *Chemicals, Plastics, and Petroleum Products*. It is supplied as a powder and used in experimental diets by mixing with Fox Chow at levels of 0.01%, 0.1%, 1.5%, and 3.0%, by weight. White rats of the Rochester Wistar strain were used.

Part A. Eighteen male weanling rats of 60 to 75 g and 18 adult male rats of 150 to 175 g were given Fox Chow with 1.5% *Flectol H* by weight. After 24 hours, daily sacrifices of 3 rats from each group were performed for the next 6 days.

Part B. Twenty male and 20 female rats of 50 to 60 g were placed on control diet and on diets containing 1.5% and 3.0% *Flectol H*. Five rats from each group were sacrificed at weekly intervals for one month.

Part C. Thirty-five male and 35 female rats were maintained for 2 years on the fol-

lowing diets: control, 0.01%, 0.1% and 1.5% *Flectol H*.

In part C, rats were weighed weekly while the others were weighed at time of sacrifice. Animals were allowed *ad libitum* access to diet and water. Rats were sacrificed by decapitation, autopsies performed, and organ weights recorded. Only 3 males and 5 females given the 1.5% diet in the 2-year study survived to sacrifice. This represented decreased survival as compared to the other groups. Tissues fixed in 20% neutral formalin were stained with hematoxylin and eosin. Selected sections were examined with polarized light and with fluorescent light microscopy. Fat stains were prepared on fixed frozen sections of liver using oil red O. PAS, iron stains, acid-fast stains, and fat stains on paraffinized sections were performed on selected sections.

Results. Gross observations. Parts A and B. Enlarged, pale, soft livers were evident within 2 or 3 days and markedly obvious after one week. The increase in hepatic weight was due in part, at least, to increased hepatic fat (Tables I and II). In weanling rats the total hepatic lipid increased 60% in 5 days while it stayed nearly constant in adult rats.* Slight growth lag was seen in the 1.5% diet group; the 3.0% rats failed to gain weight and did not survive beyond 3 weeks. *Part C.* Of the rats at the end of the 2-year study, those of the (male and female) 1.5% groups showed enlarged livers; female rats of the 0.1% diet group may also have had some liver enlargement. Three of the 5 female rats in the 1.5% diet group had one to several gross nodules in their livers. Nodules were 3 to 10 mm in diameter, yellow-gray or gray-white, granular, moderately firm, and occasionally umbilicated. Rarely, a red-brown nodule of several millimeters in

TABLE II. Part B. Liver Weight-Body Weight Ratio (Ranges).

Week	Control		1.5% diet group		3% diet group	
	♂	♀	♂	♀	♂	♀
1	.48-.60	.46-.53	.75-.81	.68-.74	.95-1.15	.79-.93
2	.48-.50	.47-.52	.74-1.01	.55-.95	.95-1.30	.89-1.32
3	.38-.63	.47-.55	.72-.86	.68-.81	1.34-1.44	1.06-1.41
4	.43-.51	.46-.48	.61-.73	.62-.77	—	—

* Determinations performed by Dr. Frances Haven.

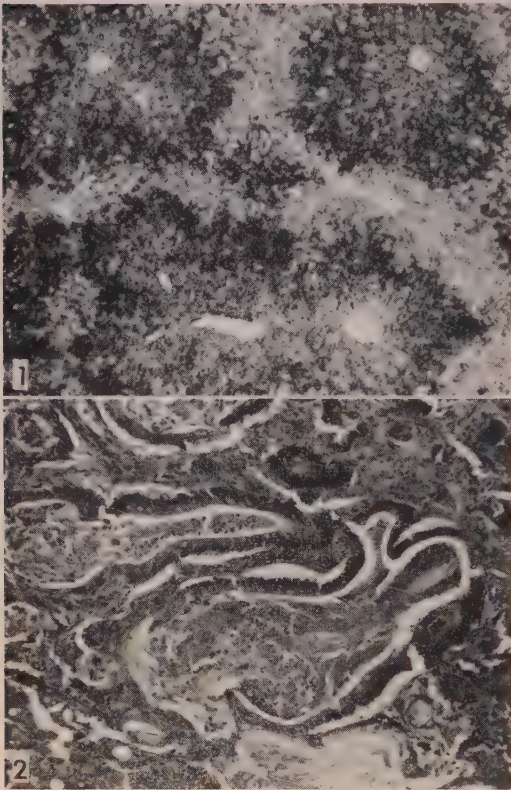


FIG. 1. Liver showing predominant midzonal distribution of fat (oil red O $\times 48$).

FIG. 2. Atypical bile-duct hyperplasia resulting from chronic administration of *Flectol H* (H & E $\times 120$).

diameter was noted. Where gross nodules were not present, whitish areas of less than one millimeter in diameter were seen in the livers of the rats on a 1.5% *Flectol H* diet. Vascular channels were frequently dilated. No cirrhosis was seen. One rat of the 0.1% diet group had numerous yellow-gray, firm, 2 to 3 mm nodules of metastatic tumor in the mesentery arising from the pancreas.

Microscopic observations. *Parts A and B.* A minute amount of fat was seen in Kupffer cells at the end of 24 hours. From the second to sixth days weanling rats showed increasing amounts of hepatic fat in midzonal and occasionally in centrolobular or periportal areas while adults showed inconstant lesser amounts of fat (Fig. 1). During the period from one week to one month the amount of fat increased as its distribution became more diffuse. Rats of the 1.5%

diet group tended to have more obvious midzonal fat while those of the 3% diet group showed severe diffuse fatty change. Cellular changes included loss of cytoplasmic basophilia, hyaline degeneration, nuclear enlargement, and increased numbers of double nuclei, all more obvious in the 3% diet group of rats. Biliary-duct hyperplasia was seen in the 3% diet group at the end of 3 weeks and at the end of 4 weeks in the 1.5% diet group. Often it was difficult to distinguish the hepatic or biliary origin of clusters of proliferating cells. One rat only exhibited bile plugs within canaliculi.

Part C. Most marked change was found in the 1.5% diet group of rats. Gross nodules corresponded to the most atypical biliary-duct hyperplasia. These consisted of irregular acini lined by columnar cells with basal nuclei and often showing goblets of mucoid material in the cytoplasm. Lumens contained mucin, eosinophilic debris, and leukocytes. Frequently, stratification of nuclei and mitoses were seen in the most atypical acini. Within the large amount of surrounding fibrous connective tissue were small regenerative islands of liver cells and numerous inflammatory cells (Fig. 2). The rest of the liver revealed smaller foci of duct hyperplasia, variation in hepatic cell size, and enlarged hepatic cells with eosinophilic cytoplasm. Globular yellow-brown pigment was prominent in both Kupffer cells and parenchymal cells. Special stains suggest that this pigment is a lipofuscin.

Discussion. Localization of fatty metamorphosis and toxic hepatic injury within the hepatic lobule may be limited to the central or periportal zones in response to a variety of agents(1,2). Rarely, as in the responses to beryllium injection(3) or to chloroform by hyperthyroid rabbits(4), fatty change and necrosis are limited to the midzone of the lobule. *Flectol H* is capable of producing midzonal localization of fat without significant hepatic parenchymal necrosis. No single hypothesis based on either circulatory or on functional differences within the lobule would seem to explain the simultaneous occurrence of central, midzonal, and periportal fatty change in response to *Flectol H*.

The biliary-duct hyperplasia and hepatic cell alterations are similar to the effects of butter yellow(5,6,7) as well as of a number of other agents(8,9). This atypical proliferation of biliary ducts and of fibrous tissue is called cholangiofibrosis and is considered to be a precursor of biliary-duct carcinoma(7). If analogy to butter yellow is extended, it is probable that dietary alterations will cause more rapid onset of these lesions.

Such reactions to feeding an antioxidant raise questions as to mechanism and site of action. Relations worthy of investigation are those involving Vit. A, Vit. E, and choline and methionine. Further work may also be of value in elucidating lobular localization of hepatic injury and the responses of biliary-duct epithelium and hepatic cells.

Summary. *Flectol H*, a polymer of 1,2-dihydro-2,2,4-trimethylquinoline, when fed to rats produced fatty change frequently lo-

calized to the midzonal area of the hepatic lobule. Chronic administration led to alteration of hepatic cells, necrosis, and atypical biliary-duct hyperplasia.

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Extratrabeular Crystallization in Rickets; Effects of Vitamin D, Calcium and Norethandrolone.*† (26223)

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Recent observations have revealed the presence of abnormal mineral deposits adjacent to the bone trabeculae of chicks fed a diet containing 50% ground seeds of *Lathyrus odoratus*(1). Another series of experiments, yet unpublished, involving suckling rats injected with the toxic agent of the lathyrus seed, Beta-amino-propionitrile, has produced similar extratrabeular masses. In both instances and also in a recently observed specimen of human osteogenesis imperfecta, the large round crystals were best seen in dark-phase microscopy; they were birefringent in polarized light. The bone trabeculae

were immature, fibrillar; they were deficient in polysaccharides and minerals. A current series of experiments involving undemineralized sections of tibiae from rachitic chicks variously treated has revealed a similar condition.

Materials and technics. Five groups of 10 newborn chicks/group were fed the low Ca AOAC diet(2) without Vit. D. After one week the diet was supplemented in 4 groups as follows: (a) $\text{Ca}_3(\text{PO}_4)_2$ 1%; (b) Vit. D³ 1000 units %; (c) Norethandrolone (Nilevar, Searle) 0.5 g/kilo; (d) Norethandrolone as in (c) with $\text{Ca}_3(\text{PO}_4)_2$ as in (a). Group 5 was unmodified. At 2 weeks of age, the untreated birds were suffering from severe rickets as judged by tarso-metatarsal radiographs(3), bone ash, Ca^{45} intestinal absorption(4) and by the histological picture of cartilage and bone. Calcium and norethan-

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FIG. 1. New periosteal bone trabeculae and extratrabecular crystals, from 12 μ celloidin section

drolone treatments have modified the histology and histochemistry of these tissues as recently recognized(5). The intimate effects of these agents on the mineral fraction are still unknown.

After 2 weeks in the present series, the chicks were given 100 μ C P³² in 0.5 ml distilled water intraperitoneally. Five chicks from each group were sacrificed 2 hours later. The right tibia was roughly dissected, fixed in 10% neutral aqueous formaldehyde embedded in celloidin and cut at 12 μ . For phase contrast microscopy, the sections were mounted in neutral synthetic resin (Permount, Fisher) without staining.

Observations. Unstained sections examined under negative phase contrast (Dark M, AO) have revealed the presence of round masses, 3-5 μ in diameter, shining bright except for a small dark core (Fig. 1). These were located along thick, dark newly-formed trabeculae (Fig. 1) and were present in the untreated rachitic group and also in all treated birds except those which received Vit. D. The brilliant objects were more numerous but of similar size in the sections from birds treated with calcium, norethandrolone or norethandrolone-Ca. In the chicks which received Vit. D, the large extratrabecular masses were generally not observed (Fig. 2). The bone trabeculae appeared denser and bright (Fig. 2). The objects thus seen in negative phase were rapidly dissolved in acid dyes; they were stained blue by azur II at pH 7.4. The Von Kossa reaction has yielded irregular concentrations of coarse precipitate between the trabeculae. These could have been interpreted as artefacts in absence of the phase contrast evidence (Fig. 1). The extratrabecular masses were birefringent in polarized light. Under the present conditions, they produced a P³² autoradiograph. Alpharadiography(5) has shown them as uniformly dense.

Discussion. The positive Von Kossa reaction and P³² autoradiography at the sites of extratrabecular globule formation, point to

of undemineralized rachitic chick tibia. Dark M phase contrast $\times 600$.

FIG. 2. A comparable area from tibia of a chick treated with vit. D³ for 1 wk. Dark M phase contrast $\times 600$.

the presence of calcium phosphate in these crystals which appear in the vicinity of trabeculae unable to mineralize in rickets as well as in osteolathyrism of the chick(1), BAPN poisoning of young rats and human osteogenesis imperfecta.

Calcium and norethandrolone help bone growth but do not favor maturation(6). Calcium and norethandrolone combined have apparently produced maturation of the organic matrix(6); the present experiments reveal however that extratrabeular crystallization is not reduced by that treatment as it is by Vit. D (Fig. 2). The present experiments indicate also that even in rickets, the local mineral deposit is fairly abundant. The numerous osteoclasts which inhabit the vicinity of the growth sites might be instrumental in local mobilization of salt in order to "make the interstitial fluid rich in calcium," a condition essential for mineralization as postulated by Leriche and Policard(7). The primary lesion of bone in rickets which is cured by Vit. D would then be related to the organic matrix. On the other hand, the in-

creased Ca^{45} uptake by bone under high Ca deposit(8) may be intimately responsible to the presence of extratrabeular crystals and not to an activation of bone growth.

Summary. Large extratrabeular crystals are present in rachitic bones of chicks. They disappear after treatment with Vit. D.

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Additional Evidence for Involvement of RNA in Maintenance of ATP Levels in Homogenates.* (26224)

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Rat liver homogenates preincubated with ribonuclease (RNase) lose the capacity to convert mevalonic acid (MVA) to non-saponifiable material (NSF) and cholesterol(1). This loss of synthetic capacity is associated with absence of ATP in these homogenates (2,3). ATP is present and conversion of MVA to NSF occurs if the homogenates are supplemented prior to or after preincubation with autoclaved liver extract, or with relatively large amounts of RNA, or with DNA, or with various anionic polymers such as polyethylene sulfonate or heparin (4,5,6). It has been concluded that a polyanion, which in

the case of whole liver is RNA or a ribonucleoprotein, is essential for maintenance of ATP levels in tissues(7,8). Inactivation of biosynthetic capacity in liver homogenates with respect to MVA conversion to NSF also is observed on preincubation with various polycations such as protamine or polylysine(9,10). The effect of these agents similarly is preventable or reversible with the same group of polyanions. These findings with polycations raise the question as to whether inactivation with RNase is attributable to its enzymatic activity or to its capacity as a basic protein to combine with or precipitate polyanions unrelated in structure to RNA. The experiments described here demonstrate that when RNase is denatured by acid and heat under condi-

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tions where enzymatic activity is largely lost without significant alteration of acid-base properties the capacity to inactivate homogenates disappears. These findings provide additional evidence that RNA is, indeed, a component of an aerobic system concerned with the maintenance of ATP levels in liver homogenates.

Materials and methods. The biosynthetic experiments involved a preliminary preincubation period where essentially whole homogenates of rat liver (1 part liver, 2 parts phosphate-magnesium-nicotinamide buffer, loose homogenizer, 200 xg supernatant fraction, aeration) were treated (30 min, 37°, agitation) with various amounts of native or denatured RNase followed by a biosynthetic period where the homogenates were supplemented with MVA-2-C¹⁴ (0.5 mg DL MVA, 60,000 cpm), aerated, and reincubated (3 hr). All flasks contained supplements of 1 mg ATP, 10 mg DPN, and 100 mg sodium succinate. Following the biosynthetic period the contents of each flask were saponified with alcoholic KOH, extracted with petroleum ether, the extracts dried with sodium sulfate, filtered, evaporated to dryness, taken up in scintillation mixture, and counted. The results given are in terms of total cpm per incubation flask. Further details of the biosynthetic experiments have been published (1-10). RNase (Schwarz, 5 x crystallized) was denatured by heating 10 ml amounts of a solution (5 mg/ml) at pH 1.0 in a vigorously boiling water bath for the time intervals indicated. Following denaturation the solutions were neutralized and used immediately in the biosynthetic experiments. Determinations of RNase activity as a measure of the extent of inactivation were carried out by the method of Kunitz (11) within 3-4 hrs of heat treatment.

Results. As indicated by Exp. 1, Table I, preincubation of liver homogenate with essentially inactivated RNase (90 min. 84% inactivation), in contrast to native RNase, is associated with no reduction in capacity to convert MVA to NSF. Similarly RNase heated for a shorter time (Exp. 2, 60 min, 72% inactivation) is not deleterious and, in fact, at intermediate levels some stimulation

TABLE I. Biosynthesis of NSF by Liver Homogenate as Influenced by Preincubation with Various Native and Heat-Acid Denatured RNase Preparations.

Exp.	RNase preparation		NSF, cpm
	Level, mg	Treatment	
1	0	Native RNase	4976
	1		1436
	2		426
	5		199
	10		132
	20		155
	1	90 min., pH 1.0, 100°, 84% inactivation	5226
	2		5180
	5		5982
	10		6228
	20		6630
2	0	Native RNase	5132
	1		790
	2		65
	5		37
	10		25
	20		21
	1	60 min., pH 1.0, 100°, 72% inactivation	7409
	2		7769
	5		8166
	10		7285
	20		7065
3	0	Native RNase	5743
	1		1186
	2		504
	5		213
	10		176
	20		280
	1	30 min., pH 1.0, 100°, 51% inactivation	12002
	2		9773
	5		3793
	10		1617
	20		1424
4	0	Native RNase	5557
	1		424
	2		124
	5		77
	10		32
	20		96
	1	30 min., pH 1.0, 100°, 38% inactivation	8208
	2		4094
	5		427
	10		169
	20		79

of MVA utilization is evident. With RNase preparations less extensively inactivated (Exp. 3 and 4, 51 and 38% inactivated according to the Kunitz assay) the phenomenon that began to be apparent with the 72% inactivated preparation *i.e.* stimulation of MVA utilization at intermediate levels is evident. The data from Exp. 3 and 4 (51 and 38% inactivation) are summarized in Fig. 1. Clearly, preincubation of liver homogenates with only partially de-

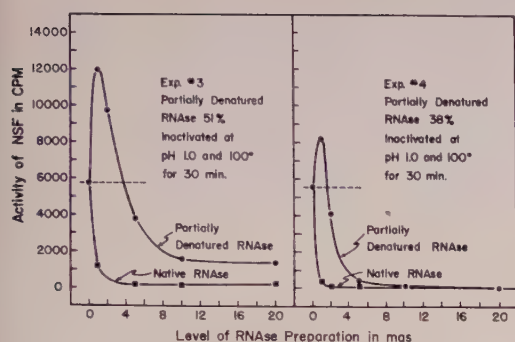


FIG. 1. Biosynthesis of NSF by liver homogenate as influenced by preincubation with native or partially denatured RNase.

natured RNase is associated with 2 superimposed effects: (1) stimulation of MVA utilization at low levels and (2) inhibition of MVA utilization at higher levels. In all experiments preincubation of liver homogenates with native RNase was associated with at least 95% reduction in capacity to convert MVA to NSF.

Discussion. The data presented demonstrate that the capacity of RNase to inactivate a system essential for conversion of MVA to NSF is associated with the enzymatic activity of RNase rather than with its basic properties. Thus it would appear that a substrate for RNase is involved in the entire liver homogenate system. Previous experiments have provided evidence that the natural factor, at least as far as liver is concerned, is an RNA or ribonucleoprotein and the locus of activity is somewhere in maintenance of ATP levels presumably either as a factor essential in respiration or oxidative phosphorylation or as an inhibitor of ATPase activity.

Person and Fine recently have reported (12) that the activity of isolated preparations of cytochrome oxidase is inhibited by a number of basic compounds including RNase and protamine. This inhibition is reversed by the strongly anionic compound polyglucose sulfate. Person and Fine conclude that basic polymers are direct inhibitors of cytochrome oxidase. If there is any analogy between the experiments of Person and Fine and those involving RNase and protamine as inactivators of a system concerned with maintenance of ATP levels of liver homogenates, it is suggested that RNase and protamine may not

inhibit cytochrome oxidase directly but do so indirectly by hydrolyzing an associated RNA in the case of RNase or by combining irreversibly with the RNA in the case of protamine. It should be noted that at least some cytochrome preparations that have been obtained in a state of relative purity are strongly basic and it is reasonable to postulate that they function in association with RNA. Possibly the integrated function of the cytochromes in electron transport is a consequence of a close and orderly arrangement on a polyanionic framework.

Some comment is in order concerning the phenomenon observed with partially denatured RNase where preincubation of liver homogenates with small amounts of this material is associated with a greater conversion of MVA to NSF, and presumably, a higher level of ATP, than encountered in controls. It is known from published and unpublished experiments in this laboratory that frequently stimulation of MVA conversion to NSF is obtained with anionic polymers in homogenates that have been preincubated without added RNase or high molecular weight basic compounds. Apparently in the preincubation of liver homogenates various "endogenous" diesterases and other enzymes sometimes reduce the level of RNA to a limiting extent. It is suggested that partially denatured RNase shows stimulation because part of it has been altered to the extent that it combines with substrate to yield a ribonucleoprotein where RNA moiety is "protected" against other enzymes but is not bound to the extent of interference with function. Obviously at higher levels of the altered material the unaltered or native portion must somehow outstrip that portion affording protection and the resultant effect is hydrolysis of RNA, loss of ATP level, and resulting failure of MVA conversion to NSF. Other interpretations of the data may be formulated.

Summary. Preincubation of liver homogenates with RNase preparations nearly completely denatured with respect to enzymatic activity by heat and acid is associated with no reduction in capacity to convert mevalonic acid to non-saponifiable material. Mevalonic acid utilization is completely eliminated by

preincubation with native RNase. The preincubation of liver homogenates with less completely inactivated RNase involves more complicated phenomena. The results are interpreted to mean that the inactivation of MVA utilization observed with native RNase is due to enzymatic activity of RNase rather than to the basic properties of the protein that are not significantly altered on mild denaturation. Additional evidence is thus provided for the involvement of RNA (or a ribonucleoprotein) in the complete system essential for conversion of mevalonic acid to non-saponifiable material by liver homogenates. Evidence has been presented previously that the RNA is essential for maintenance of ATP levels required in a number of steps involved in utilization of mevalonic acid.

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Production of Circulating Antibody against Defined Antigen (BGG) by Neonatal Guinea Pigs. (26225)

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The immunologic capacity of young guinea pigs is generally acknowledged as being considerably less than that of adults(1-5). Investigations leading to this conclusion employed chemically undefined antigens, such as sheep serum(1), diphtheria toxin(2), tubercle bacillus(3), typhus vaccine(4), and thyroid tissue(5), and the demonstrations principally concerned sensitization rather than antibody detection. On the other hand, studies of acquired tolerance, naturally induced *in utero*, toward postnatally implanted maternal skin homografts(6,7), and demonstration of plasma cells in lymph nodes of newborn guinea pigs(8) constitute indirect evidence that guinea pigs may already possess the capacity to form antibodies in neonatal stages.

The present study was undertaken to ascertain whether guinea pigs can form humoral antibody against defined antigen, bovine gamma globulin (BGG), immediately after birth.

Methods. Guinea pigs of outbred stock were inoculated 6 to 30 hours after delivery. Sensitization was accomplished by single intramuscular injections of BGG* (25 mg/100 g body weight) combined with Freund adjuvant.[†] Animals were bled by intracardiac puncture or challenged for anaphylaxis by intracardiac or intravenous injection. Sera were stored at -19°C.

The passive cutaneous anaphylactic (PCA) procedure, as developed by Ovary (10), was employed for detection of circulating antibody; the method is capable of demonstrating precipitable and nonprecipitable antibody and is sensitive down to the order of 0.003 μ g antibody nitrogen(9,10). Intracutaneous injection of 0.1 ml undiluted antiserum was followed, 5 to 6 hours later, by

* Bovine gamma globulin, fraction II, from Nutritional Biochemicals Corp., Cleveland, O.

[†] Freund adjuvant, complete, from Difco Laboratories, Detroit, Mich.

TABLE I. PCA Reactions of Sera of Guinea Pigs Injected with BGG and Adjuvant 6 to 30 Hr after Birth.

No.	Body wt, g	Post-inj. age of serum (days) *			
		7	9	11	15
661	86	+++			
662	77	0			
663	90	0			
676	94	0			
677	91	0			
678	95	0			
703	84	0			
704	74	0			
705	79	0			
706	67	0			
710	91	0		++++	
711	88	0		++++	
712	95	0		0	++
716	106	0		++++	
717	105	0		++	++++
868	101		++		
869	78		++		
870	79		0		
871	84		0		
872	85		0		
873	79		++		
874	107		+		
734	99		++		+++
735	106		±		++
736	95		++		+++
793	78		++	++	
794	92		++	+++	
795	85		+++	+++	
796	70		++	++++	
797	70		+		
798	91			++++	
799	73			++++	
800	90			++++	
802	130			++++	
803	118			++++	
791	114			++++	++++
792	124			++++	++++
723	84				++
718	107				+++
719	96				++++
720	84				+++
729	—				+++
730	—				++++
732	—				+++
726	—				++
727	—				++
728	—				+++

* Quantitatively scored according to diameter (mm) of cutaneous reaction: 0, negative; ±, 1-5; +, 6-10; ++, 11-15; +++, 16-20; +++++, >20.

intravenous injection of 0.1 ml (10 mg) BGG in saline plus 0.5 ml of 0.5% Evans Blue solution. Cutaneous reaction appeared within 5 minutes and persisted; scoring index (Table I) was only slightly modified from that of Ovary(10).

Agar-diffusion tests were performed on

neonatal and adult sera according to the Ouchterlony procedure(11). Systemic anaphylaxis, using 13 to 25 mg BGG in saline as challenging dose, was induced in a small series of animals. Plasma-cell studies were carried out on lymph nodes and spleen, treated in accordance with the triple stain technic of Trevan and Sharrock(12).

Results. Injection of newborn guinea pigs with BGG and adjuvant on the day of, or immediately following, birth induced formation of specific antibody detectable infrequently as early as the seventh day (1/15) and generally by the ninth day (12/15) (Table I). Titers increased with age to the eleventh and fifteenth day after inoculation. No cross-reactivity occurred when bovine serum albumin (BSA) was employed for antigenic challenge or when normal (control) guinea-pig serum or anti-BSA serum was tested against BGG. Percentages of positive PCA reactions with sera of different postinjection intervals are shown in Fig. 1.

Agar-diffusion tests on these neonatal sera gave negative results. Ouchterlony tests on adult sera, on the other hand, gave positive reactions 9 days after single intramuscular injections of BGG and adjuvant.

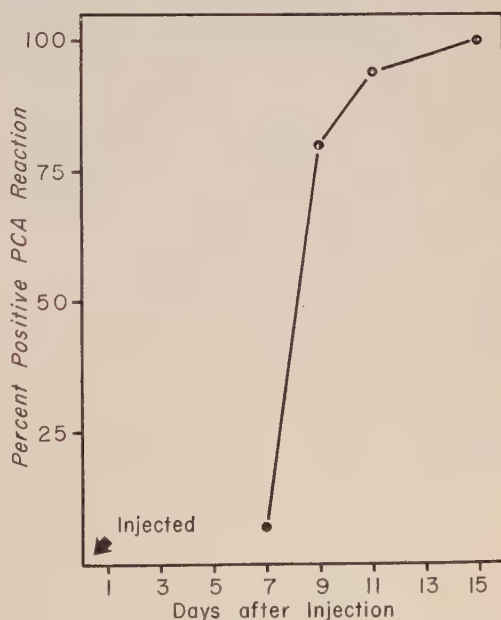


FIG. 1. Positive PCA reactions of neonatal guinea-pig sera after inj. with BGG and adjuvant at birth.

TABLE II. Systemic Anaphylaxis in Newborn Guinea Pigs Sensitized toward BGG Injected with Adjuvant.

Animal No.	Inj. age (days)	Sensitization dose (mg/100 g b.w.)	Challenge		Reaction*
			Age (days)	Dose (mg)	
661	2	29	12	25	+++
662	2	32	9	13	+
663	2	28	9	25	+
798	1	25	12	20	+
799	1	25	12	20	++
802	1	25	12	20	+++
803	1	25	12	20	+++
829	2	25	9	20	+
830	2	25	9	20	+

* Reaction: +, mild; ++, severe; +++, lethal.

Neonatal guinea pigs, inoculated at 1 or 2 days of age, suffered systemic anaphylaxis when challenged at 9 or 12 days of age (Table II). At day 12, sensitization was sufficient to cause fatal reactions. No unfavorable response occurred when BGG-sensitized animals were challenged with nonspecific antigen (BSA).

Plasma cells and plasma-cell precursors were observed in methyl green-pyronin-orange G preparations of lymph nodes from normal late prenatal, neonatal, and adult animals. The presence of these cells in newborn guinea pigs confirms the findings of Gyllensten(8). No attempt was made to determine the effect of inoculation on plasma-cell population.

Discussion. Detection of induced anti-BGG antibody in newborn guinea pigs by PCA but not by the agar-diffusion procedure may be attributed to extreme sensitivity of the former method in demonstrating low concentrations of precipitable antibody, without necessarily invoking the possibility that the detectable antibody is of the nonprecipitable type. Since even the adult guinea pig is a relatively poor producer of precipitable antibody, compared, for example, with the rabbit(9), low antibody titers would be expected in neonatal guinea pigs.

Until antibody production by fetal guinea pigs is unequivocally demonstrated, some question will exist as to whether this capacity is limited to postnatal stages or is established at a reduced rate in the late prenatal

period, prior to parturition. In the above series (Table I), approximately 9 days were required to develop a fair titer of antibody and 11 days to produce a relatively high titer. In 2 groups of neonatal guinea pigs injected on the fourth and sixth days of age, antibody concentration was moderate after 7 days and high after 9 days. In adults, strong PCA reactions have indicated high-titer sera 7 to 8 days from time of injection. It would appear then that antibody-production capacity is increasing during the first week and may be present to a degree before birth.

The results indicate that attempts to induce effective tolerance in the guinea pig toward skin homotransplantation, or other forms of immune reaction, must be initiated in the fetus. They also clarify the immune status of neonatal guinea pigs subject to induced aspermatogenesis, as discussed elsewhere(13).

Summary. Neonatal guinea pigs, injected with BGG and adjuvant immediately after birth produce circulating antibody, detectable by PCA but not by agar-diffusion tests. Sera collected 7, 9, 11, and 15 days after intramuscular injection gave 7, 80, 94, and 100% positive PCA reactions, respectively. Rate of antibody production during the first week after birth is lower than that of adults. Capacity for humoral antibody formation probably exists at a reduced level in the fetus.

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Observations on Mechanism of Potassium Excretion due to Administration of Hydrochlorothiazide. (26226)

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Administration of thiazide diuretics to normal subjects and to patients with compensated congestive heart failure produced only moderate urinary excretion of potassium (K)(1,2). On the other hand, these agents induced much greater kaliuresis in patients with edema and ascites(3-6). This difference suggested to us that aldosterone may influence K loss due to the thiazide diuretics inasmuch as most patients with significant edema and ascites excrete abnormally large quantities of aldosterone in the urine(7). This hormone is believed to promote renal excretion of K only when there is an adequate sodium (Na) transport to the distal tubular site for Na and K exchange (8-10). The present studies in laboratory animals represent an attempt to clarify the influence of aldosterone-like steroids on K excretion with thiazide diuretics.

Method. Holtzman rats, 150-200 g, were housed in a constant environment and fed Purina chow biscuits and tap water for 4 days prior to study. Adrenalectomy was performed through a dorsal incision under ether anesthesia and the animals were then maintained on sugar and tap water postoperatively. Approximately 24 hours after adrenalectomy the animals were injected subcutaneously with 2.5 ml of 0.9% saline and with desoxycorticosterone (DOC) as the acetate form in 0.25 ml of ethanol-isotonic saline solvent (28/72 by volume). DOC was used as a standard aldosterone-like steroid(13) because of its availability. Hydrochlorothiazide (H-C) was given subcutaneously in 0.25 to 0.5 ml of ethanol-saline, either alone or in combination with DOC at the time of the sa-

line load. Control injections of solvent were used where indicated. Animals were placed in metabolism cages (4 rats/cage) for collection of a 4-hour sample of urine. Bladders were emptied by suprapubic pressure before and at the end of collection. Urinary sodium and potassium were determined by flame photometric analysis. Statistical comparisons were done by Students "t" test.

Results. Table I summarizes the effects of DOC and H-C on Na and K excretion in adrenalectomized rats. DOC alone at doses of 3, 6, and 24 μ g induced a strong retention of Na with only slight insignificant changes in urinary excretion of K. H-C significantly increased urinary output of both Na and K at doses of 0.6 and 1.2 mg/kg ($P < 0.05$). There was no significant increase in potassium excretion with the 0.3 mg/kg dose when

TABLE I. Urinary Excretion of Na and K Following Treatment with Desoxycorticosterone and Hydrochlorothiazide in Adrenalectomized Rats.

Treatment			Mean response	
DOC, μ g	H-C, mg/kg	No. of measurements	Na	K
—	—	8	.69 $\pm$.23	.37 $\pm$.09
3	—	11	.36 $\pm$.19*	.46 $\pm$.15
6	—	9	.18 $\pm$.06*	.42 $\pm$.14
24	—	4	.12 $\pm$.04*	.38 $\pm$.03
—	.3	4	.93 $\pm$.15	.47 $\pm$.07
—	.6	5	1.21 $\pm$.25*	.61 $\pm$.15*
—	1.2	5	1.28 $\pm$.30*	.62 $\pm$.09*
3	.3	3	.78 $\pm$.07	.76 $\pm$.07†
6	.3	4	.56 $\pm$.13	.75 $\pm$.15†
3	.6	6	1.08 $\pm$.17	.72 $\pm$.03
6	.6	4	.75 $\pm$.19	.70 $\pm$.11
24	.6	4	.66 $\pm$.11	.94 $\pm$.09†

* $P < 0.05$ relative to untreated controls.

† $P < 0.05$ relative to corresponding treatment with hydrochlorothiazide (H-C) above.

Na and K = mean meq output/4 hr $\pm$ stand. dev. Each measurement = 4 animals.

compared with the untreated control ($P > 0.05$ – < 0.10). In the presence of DOC, however, K excretion with 0.3 mg/kg of H-C was significantly augmented relative to the corresponding response to thiazide or to DOC alone. The same doses of DOC when given with 0.6 mg/kg of H-C did not further increase K excretion. However, when the dose of DOC was increased to 24 μ g further K loss occurred. The Na losses induced by H-C were effectively reversed by small doses of DOC as shown in the table. There was progressive reduction of Na excretion without an increase in K excretion when the animals were given increasingly larger doses of DOC alone, suggesting that the steroid promoted reabsorption of Na directly in addition to promoting Na-K exchange, and that this mechanism for direct reabsorption is proximal to the Na-K exchange site.

Discussion. Results of our studies suggest that one of the major mechanisms of K excretion following administration of H-C may be the presence of aldosterone-like steroids. The K loss in this situation is believed to result from inhibition of Na reabsorption in the proximal renal tubule by the thiazide diuretic(11) and the resultant delivery of a larger load of Na to the distal tubule, the site of action of aldosterone-like steroids for Na-K exchange. The increase in K excretion with larger doses of H-C alone may be due to carbonic anhydrase inhibition in the renal tubular cells(11,14), or to the delivery of a larger load of Na to the distal tubule where Na-K exchange will occur even in absence of mineralocorticoid hormone.

Since H-C was used in approximately the dosage range for man, on a weight basis, it is possible that K loss seen clinically with the thiazides is a partial response to the presence of aldosterone and not due to the pharmacological effect on the kidney. Some support for the concept is gained by reports of the ability of aldosterone antagonists, e.g., steroidal spiro lactones, to prevent the K loss

resulting from administration of thiazide diuretics(12,13).

These data suggest that urinary electrolyte response to diuretic agents can be markedly influenced by the presence of aldosterone-like hormones. Endogenous production of aldosterone should be taken into consideration in the study of these agents.

Summary. Potassium excretion with hydrochlorothiazide in *adrenalectomized* rats was significantly influenced by the presence of small amounts of DOC. Hydrochlorothiazide alone caused kaliuresis only at relatively large doses. These studies demonstrate that K loss with thiazide diuretics is in part dependent upon body fluid titers of aldosterone-like steroids.

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Passive Cutaneous Anaphylaxis in the Guinea Pig as Related to the Response of Isolated Ileum. (26227)

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Passive cutaneous anaphylaxis (PCA) has become in recent years a frequently utilized tool in investigation of various immunological problems, such as detection of small amounts of antibody or antigen (reviewed in 1), competitive fixation of normal gamma-globulins* and antibodies(2), the mechanism of reversed passive anaphylaxis(3), demonstration of auto-antibodies(4,5), differentiation between isophile and heterophile antibodies to sheep erythrocytes(6), study of the role of complement in anaphylaxis(7), the reaction of systemic lupus erythematosus with DNA (8), study of antibody-hapten interactions *in vivo*(9) and others.

In fact, the easily performed PCA technic (Ovary test) tends to replace the more elaborate Schultz-Dale test, usually regarded as the most reliable procedure for quantitative assay of anaphylactic sensitization. It was therefore decided to investigate the relative merits of both tests in parallel experiments with guinea pigs sensitized by known amounts of antibody or antigen. The investigation has confirmed the reliability of the PCA technic both in direct and reversed passive anaphylaxis.

Materials and methods. Albino guinea pigs of 200 ± 20 g were used throughout the experiments. The antigens used were: a) for direct passive anaphylaxis, chicken egg albumin (Ea) 3 times crystallized, prepared according to the method of Kekwick & Cannan (cf. 11, p 448); b) for reversed passive anaphylaxis, rabbit gamma globulin (RGG) as prepared by Kendall (cf. 11, p. 461).

* The ability of normal rabbit gamma-globulin to inhibit PCA reactions to rabbit antibody (Ovary and Bier (2)) led us to investigate the possibility of producing reversed PCA by using rabbit gamma-globulin as an antigen (Bier and Siqueira(3)). This was done without knowledge of the work of Van den Ende(10), which only came to our attention after paper(3) was published.

Guinea pigs were immunized against RGG with the aid of Freund's adjuvant. The pooled guinea pig antisera titrated 0.03 mg AbN per ml. Anti-Ea was prepared in rabbits and titrated 0.400 mg AbN per ml.

Ovary test. In direct PCA, amounts of anti-Ea ranging from 10 to 100 mcg AbN were injected intravenously and followed at intervals of 2, 6, and 24 hours, by intradermal injection of 0.1 ml of adequate dilutions of Ea. In reversed PCA experiments, 0.500 mg RGG were injected intravenously followed 2-120 hours later by 0.1 ml of adequate dilutions of guinea pig anti-RGG. In either case, intradermal injections were immediately preceded by intravenous injection of 0.5 ml of 1% Geigy Blue in saline. Animals were sacrificed 15 minutes after intradermal challenge and the diameter of the reaction was measured on the internal side of the skin.†

Schultz-Dale test. Ileum segments of guinea pigs injected intravenously with anti-Ea or RGG, as indicated above, were tested in 10 ml of Tyrode's solution in a smooth muscle bath. 0.1 ml of adequate dilutions of Ea or anti-RGG was added to the bath and the intensity of contraction was compared to that provoked by addition of 0.2 ml of a 1:5000.000 dilution of histamine expressed as base. Results were recorded as +, ± or 0, a + result corresponding to a contraction equal or greater than elicited by the histamine standard.

Results. The results obtained are recorded in Tables I and II.

Discussion. In guinea pigs sensitized by intravenous injection of antigen (RGG) or antibody (rabbit anti-Ea) and challenged with the other reactant after adequate latency periods, a reasonable degree of parallelism was found between PCA response and

† In some experiments results were recorded as ± (less than 5 mm), + (5-10 mm), ++ (10-15 mm), and +++ (15-20 mm).

TABLE II. A Comparison of Ovary and Schultz-Dale Tests in Reversed Passive Anaphylaxis. Guinea pigs injected intravenously with 0.5 mg RGG N and challenged with anti-RGG after different latency periods.

Latency period, hr	Ovary test with intraderm. inj. anti-RGG:				Schultz-Dale test after addition of anti-RGG:	
	1:50	1:100	1:200	1:500	1:1	1:10
2		±	0	0	0	0
		±	±	0	0	0
		±	±	0	0	0
		+	+	0	0	0
		+	±	0	+	0
4		++	+	±		
		++	++	++		
		+	+	+		
		++	++	±		
6		+	+	±	+	+
		++	+	±	+	+
		++	+	±	+	+
		++	+	0	+	0
8		++	++	±	+	0
		++	+	±		
		+++	+++	+++		
		+++	+++	+++		
24	+++			+	+	+
	++			±	+	+
	++			±	+	+
					+	0
48	+++			++	0	0
	++			+	0	0
	++			±	+	0
					0	0
					0	0
					+	0
					+	0
72			++		+	0
			++		+	+
			+		+	0
					0	0
96		±	0		0	0
		+++	+++		0	0
		++	+		0	0
		+++	++		+	+
120		++	+			
		++	±			
		±	±			
Controls not inj. with RGG		—	—	—		
		±	±	—		
		—	—	—		
		—	—	—		
		±	—	—		
	±			—		
	±			—		
	—			—		
	—			—		

the classical Schultz-Dale test (Tables I and II). This finding seems to indicate that in passive reverse anaphylaxis when the intra-

venous route is used for sensitization a common mechanism is involved which accounts for the local accumulation of dye and for the contraction of smooth muscle.

As pointed out by *Ovary*(3b), the severe and sometimes fatal reaction observed after successive intravenous injections of antigen and antibody may be ascribed to the action of toxic antigen-antibody complexes and eventually to the formation of anaphylatoxin. While these cases may not really correspond to passive reverse anaphylaxis, experiments such as recorded in Table II indicate that previous fixation of the gamma globulin antigen is an essential prerequisite for sensitization, as pointed out by *Van den Ende* and recently confirmed by others (3a, 3b, 3c, 12).

In the present experiments, dye accumulation after intradermal challenge proved to be a more sensitive test for detecting sensitization after too short or too long latency periods, particularly when a small amount of antibody was used, as shown in Table I for 10 µg AbN and a latency period of 2 hours, and in Table II for a latency period of 96 hours. Under these circumstances, minimal amounts of either antibody or antigen are revealed by PCA which would be undetected or only irregularly detected by the more cumbersome Schultz-Dale technic.

Summary. Guinea pigs were sensitized by intravenous injection of known amounts of antibody or antigen and challenged after varying latency periods with the other reactant. A comparison of the results obtained by local accumulation of dye after intradermal challenge (Ovary test) and by the classical Schultz-Dale test showed that the former behaves as a more sensitive test, particularly when small amounts of antibodies or antigen are involved and after inadequate latency periods. It is therefore justifiable to use Ovary's test as a reliable substitute for the Schultz-Dale technic in qualitative and quantitative assays of anaphylactic sensitization.

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Dephosphorylation of Psilocybin to Psilocin by Alkaline Phosphatase. (26228)

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A number of indolic compounds have gained pharmacological and biochemical interest because of their ability to produce psychotic states in human subjects. Thus, compounds such as bufotenin, N, N-dimethyltryptamine and harmine(1) have been reported to produce behavioural changes in man and various central nervous system effects in experimental animals. The most recent additions to this class of compounds are the substances found in the *Psilocybe mexicana* Heim mushroom, psilocybin and psilocin. Chemically, these compounds are 4-phosphoryl-N, N-dimethyltryptamine and 4-hydroxy-N, N-dimethyltryptamine, respectively. Their chemical structures are shown below.

According to Troxler *et al.*(2) psilocybin and psilocin are the first indole compounds of natural sources possessing substitutions on the 4-position of the indole ring. Psilocin differs from bufotenin only by having its -OH group located on the 4- rather than the 5-position.

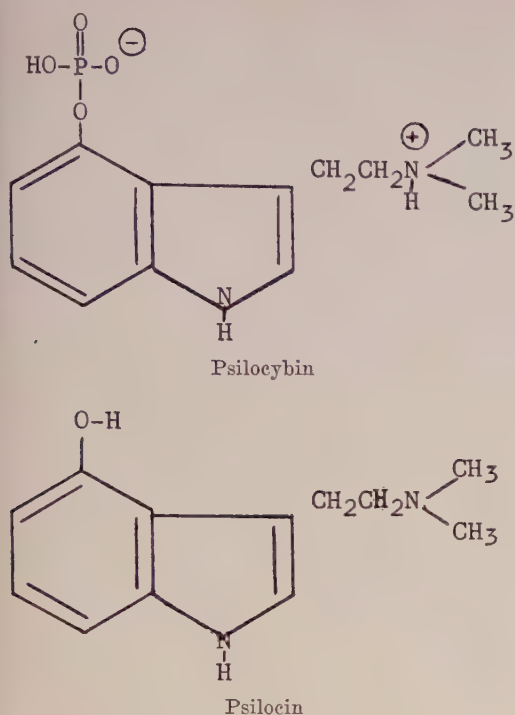
The present report is concerned with hydrolysis of the phosphate group of psilocybin *in vitro* by a purified preparation of calf intestinal phosphatase. These studies were initiated because of the possibility that psilocy-

bin might serve as a substrate for the phosphatase enzyme. This appeared reasonable since other aromatic compounds with a phosphate group are excellent substrates of acid and alkaline phosphatases(3).

Methods. All experiments in this study were carried out using purified calf intestinal phosphatase containing 15,000 units per mg.* Psilocybin and psilocin were generously supplied by Mr. Harry Althouse of Sandoz Pharmaceuticals, San Francisco, Calif.

Inorganic phosphate was determined according to a modified method of Fiske and Subbarow(4). Psilocin was determined by adapting the colorimetric analysis for serotonin as described by Udenfriend *et al.*(5). All steps in the procedure were similar to that for serotonin. The final reaction between the nitrosonaphthol reagent and psilocin in an acid medium resulted in formation of a brownish-orange color as compared to the bright violet color of serotonin or bufotenin. The color with psilocin exhibited maximum absorption at wave lengths of 540 and at 430 m μ as read on the spectrophotometer. Determinations of psilocin were made at 430

* Purified alkaline phosphatase preparation was purchased from Mann Research Labs., New York.



μ , although in some instances they were also checked at 540μ . Colors developed from standard solutions of pure psilocin were found to follow Beer's Law within the concentration range of 0.02 and 1.0μ moles/ml.

Psilocybin also reacted with the nitrosonaphthol reagent but only at much higher concentrations. It exhibited a light yellow color, but this did not interfere with the psilocin readings at 430μ . Psilocybin, however, is not extracted into the butanol phase, and the final acid extract did not give any color reaction with the nitrosonaphthol reagent. This permits a selective extraction of psilocin by the butanol and prevents interference by any psilocybin that might be present.

The incubation mixture consisted of the following: 0.4 ml of intestinal phosphatase (1500 units/ml) and 5.0 ml of psilocybin (2μ moles/ml) dissolved in Veronal buffer (pH 9.2). The psilocybin was pre-incubated for 5 minutes in order to equilibrate to 37°C . The enzyme solution was then added, and at varying times, tubes were removed and assayed for psilocin and inorganic phosphate. Duplicate assays were performed for each tube.

Descending paper chromatography of the incubation mixtures was used as a further identification of psilocin. A butanol-acetic acid-water (4:1:1) mixture acted as the developing solvent, and the spots were detected with a cinnamaldehyde-HCl reagent(6).

Results. The ability of purified intestinal alkaline phosphatase to dephosphorylate psilocybin with liberation of psilocin and inorganic phosphate is illustrated graphically in Fig. 1. A rapid dephosphorylation is evident in the initial 15-30 minutes and thereafter proceeds at a slower linear rate. Under the conditions described here it appears that about $5 \times 10^{-4} \mu$ moles of psilocin was liberated per unit of enzyme per hour. In contrast, dephosphorylation of disodium monophenylphosphate by alkaline phosphatase liberates about twice as much phenol and inorganic phosphate. In all instances liberation of inorganic phosphate follows closely the appearance of psilocin during incubation period.

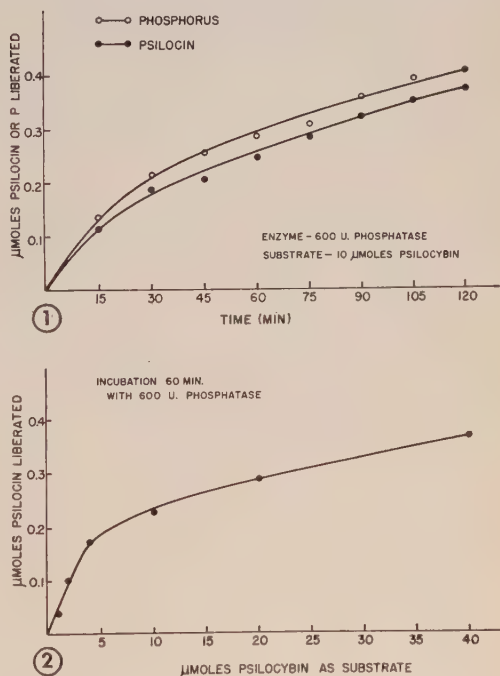


FIG. 1. Rate of phosphorus and psilocin liberation from action of purified alkaline phosphatase acting on psilocybin. Incubation in veronal buffer, pH 9.2 at 37°C .

FIG. 2. Effect of psilocybin concentration on liberation of psilocin by purified alkaline phosphatase. Incubations were carried out in veronal buffer, pH 9.2 at 37°C for 60 min.

That psilocin was liberated from psilocybin by phosphatase was evident not only from the colorimetric tests but also from the Rf values by paper chromatography. Development of the paper by spraying with the cinnamaldehyde-HCl reagent revealed only one spot identical in position with the standard psilocin strip. Average Rf value of both standard and unknown strips was 0.74 (average value of 4 strips). Psilocybin did not react with the reagent to give distinctive spot test.

The effect of psilocybin concentration on formation of psilocin is shown in Fig. 2. In these instances the incubation was carried out for one hour with 600 units of alkaline phosphatase. Concentrations of 1 μ mole to 40 μ moles of psilocybin were employed as the substrate. A linear relationship between psilocybin concentrations and psilocin formation was noted up to about 4 μ moles of substrate. Beyond this concentration, rate of formation of psilocin approached a plateau, indicating saturation of the enzyme with excess substrate and attainment of a zero-order reaction.

Since the Mg^{++} ion is known to activate alkaline phosphatase its effect was also studied with psilocybin as substrate. It was found that at concentrations of 0.35% $MgSO_4$, psilocin and inorganic phosphate liberated increased about 30% above controls.

Discussion. Psilocybin is readily dephosphorylated by intestinal phosphatase to form inorganic phosphate and psilocin. This raises the question of whether the central nervous system actions of psilocybin are produced by its dephosphorylated congener, since many mammalian tissues as well as serum contain a variety of phosphatases. Experiments in progress indicate that both mammalian tissue and plasma are capable of rapidly dephosphorylating psilocybin.

It would appear reasonable that psilocin might produce central nervous system effects of psilocybin since the latter would be highly ionized and less likely to penetrate the blood-brain barrier. Determinations of the solubilities of these 2 compounds in organic solvents and in Tyrode solution at pH 7.4 showed psilocin to be over 100 times as soluble in chloroform than was psilocybin (unpublished results).

Liberation of psilocin from psilocybin was readily determined by specific assay methods. Although closely related in structure, these 2 compounds reacted quite differently to various color reagents. We are currently exploring the possibility of utilizing the spectrofluorimeter as a means of identifying small amounts of both of these compounds.

Summary. Incubation of psilocybin with purified intestinal phosphatase resulted in liberation of psilocin and inorganic phosphate. Psilocin formed was determined both quantitatively by a specific colorimetric method employing the nitrosonaphthol reagent and qualitatively by paper chromatography. Some characteristics of the phosphatase action on psilocybin are described. The possibility of a similar reaction occurring in the intact animal is discussed.

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Influence of the Kidney on Electrolytes of the Aorta.* (26229)

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Previous investigations from this laboratory have demonstrated a positive correlation between potassium concentrations of arterial tissue and blood pressure levels(1). The increased aorta potassium content in renal hypertensive rats, first shown by Tobian and Binion(2), was considered to be a specific response to the ischemic kidney.

In the following experiment, an attempt was made to clarify the influence of the kidney on potassium content of the aorta in relationship to blood pressure. The effects of nephrectomy and ureteral tie on electrolytes of the aorta and blood pressure in hypertensive rats were studied for this purpose.

Methods. Six-weeks-old male Long-Evans rats were subjected to a modification of Grollman's method for inducing renal hypertension(3), *i.e.*, uninephrectomy and a figure-of-eight ligature around the remaining kidney. In a group of rats which exhibited significant hypertension 6 to 8 weeks later, the ligatured kidney was excised. After approximately 3 hours, blood pressures were obtained(4) and the animals then sacrificed. Blood, heart, skeletal muscle and aorta were removed for analyses of sodium and potassium as previously described(5).

A second group of hypertensive rats were subjected to ureteral ligation of the ischemic kidney. Three hours later, blood pressures were determined and the animals sacrificed for similar analyses.

A number of control groups were also studied, consisting of normal rats with bilateral nephrectomy, bilateral ureteral ligation, and sham operated by renal manipulation only. Two groups of rats subjected to the figure-of-eight renal ligation but which remained "normotensive" were also included. One group was nephrectomized and the other had a ureteral ligation performed. These rats as well as the normal controls were examined for blood pressure changes 3 hours

after operative procedure, then sacrificed for analysis of electrolytes of similar tissues.

Results. In Table I are presented results obtained from the intact rats. In rats of all groups, nephrectomy, ureteral ligation and renal manipulation, there was a small decline in blood pressure. There was also a slight decrease in sodium and potassium content of the aorta in nephrectomized and ureteral ligated rats as well as a slight decrease in myocardial potassium. Sodium content of the skeletal muscle of the operated rats was significantly increased.

Table II shows the results in hypertensive and "normotensive" rats. Nephrectomy induced a marked fall in blood pressure of hypertensive rats (70 mm Hg) and only an insignificant decrease (12 mm Hg) in hypertensives with ureteral ligation. There was relatively little change in blood pressure level of "normotensive" rats subjected to these operative procedures.

There was a marked fall in blood pressure to normal levels in the hypertensive rats following nephrectomy, associated with a decrease in aorta potassium content to normal from the originally elevated values. Aorta potassium content and blood pressure remained at elevated levels in hypertensive rats subjected to ureteral ligation. There was little change in aorta sodium content of either group.

In the "normotensive renal" ligated rats the elevated aorta potassium content fell to normal values following either nephrectomy or ureteral ligation. There was also a decrease in aorta sodium content in these animals similar to that observed in the intact rats.

Electrolytes of the myocardium remained unchanged after nephrectomy or ureteral ligation in both hypertensive and renal ligated "normotensive" animals. Skeletal muscle showed an increase in sodium content, as in the intact rats of Table I, but there was also an increase in potassium content in the hy-

* Aided by a grant from N.I.H.

TABLE I. Nephrectomy and Ureteral Ligation on Electrolytes and Blood Pressure of Normal Rats.

Type of rat	No.	Avg origi- nal B.P., mm Hg	Aorta*		Myocardium*		Skeletal muscle*		Serum		Avg post- op. B.P., mm Hg	Change in B.P., mm Hg
			Na	K	Na	K	Na	K	Na	K		
Normal	12	116 (104-124)	273 ± 5.1†	111 ± 2.0	172 ± 4.8	317 ± 2.8	105 ± 3.8	394 ± 3.6	146 ± 3.1	5.2 ± .18		
Normal (sham operation)	8	113 (102-122)	254 ± 5.8	110 ± 1.8	170 ± 6.0	314 ± 3.1	100 ± 5.1	379 ± 4.0	143 ± 2.9	5.9 ± .27	104 (94-116)	-8
Normal, neph- rectomy	10	112 (106-118)	236 ± 6.7	100 ± 3.1	168 ± 5.4	289 ± 2.6	126 ± 4.3	385 ± 3.6	144 ± 3.4	5.9 ± 1.1	100 (90-110)	-12
Normal, ure- teral tie	9	108 (102-122)	238 ± 7.2	104 ± 2.6	167 ± 6.9	294 ± 3.1	128 ± 5.0	392 ± 4.8	143 ± 3.2	6.1 ± 1.4	102 (86-112)	-6

* Fat-free dry wt.

† ± stand. dev.

TABLE II. Nephrectomy and Ureteral Ligation on Electrolytes and Blood Pressures of Renal Ligated Rats.

Type of rat	No.	Avg origi- nal B.P., mm Hg	Aorta*		Myocardium*		Skeletal muscle*		Serum		Avg post- op. B.P., mm Hg	Change in B.P., mm Hg
			Na	K	Na	K	Na	K	Na	K		
Fig. 8, hyper- tensive	14	186 (160-212)	280 ± 7.3†	131 ± 3.6	161 ± 4.9	294 ± 4.2	100 ± 3.1	379 ± 2.6	141 ± 1.4	5.2 ± .20		
Fig. 8, hyper- tensive, neph- rectomy	14	192 (156-220)	266 ± 6.3	110 ± 2.2	167 ± 5.1	298 ± 2.6	127 ± 2.0	408 ± 4.9	143 ± 1.8	6.6 ± .30	122 (104-130)	-70
Fig. 8, hyper- tensive, ure- teral tie	12	184 (158-210)	270 ± 8.4	126 ± 3.1	177 ± 4.2	296 ± 2.9	124 ± 3.0	413 ± 3.8	142 ± 1.1	6.5 ± .41	172 (140-192)	-12
Fig. 8, normo- tensive	11	128 (110-134)	260 ± 6.7	122 ± 2.9	167 ± 5.6	295 ± 3.8	107 ± 3.3	376 ± 2.1	139 ± 2.4	5.3 ± .22		
Fig. 8, normo- tensive, neph- rectomy	12	126 (108-132)	241 ± 7.1	108 ± 1.9	172 ± 4.9	297 ± 4.0	125 ± 3.4	406 ± 4.1	142 ± 1.3	6.3 ± .41	116 (94-124)	-10
Fig. 8, normo- tensive, ure- teral tie	8	122 (110-128)	240 ± 6.9	109 ± 2.1	170 ± 5.9	288 ± 3.6	120 ± 3.0	386 ± 5.4	148 ± 3.4	6.4 ± .32	124 (102-142)	+2

* Fat-free dry wt.

† ± stand. dev.

pertensive rats subjected to either nephrectomy or ureteral ligation.

Discussion. There are a number of changes in electrolyte content of the various tissues of the experimental rats. It is apparent that most of them are probably the result of urinary retention since they were induced either by nephrectomy or ureteral ligation. For the purpose of this presentation, there seems little need to discuss them in detail.

There is a distinct difference between the effect of nephrectomy and ureteral ligation on aorta potassium content of the hypertensive rats. Following removal of the ischemic kidney, potassium content of the aorta falls promptly to normal values along with the marked decline in blood pressure. Hypertensive rats with the ureteral ligature show neither a change in aorta potassium content nor blood pressure. The effect of nephrectomy on loss of potassium from the aorta appears to be a specific response since in the same animal there is an increase in skeletal muscle potassium content.

It would appear therefore that removal of the ischemic kidney eliminates a substance elaborated by this organ which induces an elevation in aorta potassium content in association with hypertension. It remains to be demonstrated whether or not this is a pressor substance. These results add to the evidence that potassium content of arterial tissues has

a positive correlation with blood pressure levels. As has been previously discussed, this cation may influence blood pressure level through altering arterial tone(1).

The evidence pertaining to the role of potassium in regulation of blood pressure is quite scanty in comparison to that of sodium. The relationship of arterial tissue electrolytes to blood pressure was recently reviewed extensively by Tobian(6), who stated "the level of potassium correlates somewhat better with blood pressure than the level of sodium."

Summary. Removal of the ischemic kidney of hypertensive rats but not ureteral ligation, induces a marked fall in blood pressure and a significant decrease in aorta potassium content. It is suggested that the ischemic kidney elaborates a substance which induces hypertension and also increases potassium content of arterial tissue.

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Differences in Thyroid Function of Several Strains of Mice.*† (26230)

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The reaction of the thyroid to an antithyroid drug differs in intensity in different strains of the same animal species. Thioura-

cil administration increases thyroid weight in some strains of mice more than in others (1,2). Wilson(3), using increase of thyroid epithelium and depletion of colloid as criteria, found considerable differences in degree of histological reaction between different strains of mice after feeding a semisynthetic diet with 0.5% sulfadiazine. The strongest reaction was found in the C₃H/Wi and BUB/Wi mice, a less strong reaction in

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the YBR/HeWi strain, a still smaller reaction in the BUC/Wi strain, and the least reaction in the C₅₇BL/Fn strain.

The secretion rate of thyroid hormone differs also in different strains of mice. Hurst and Turner(4), using Dempsey and Astwood's goitrogen technic, found a higher secretion rate in Schwing albino mice than in Rockland mice. By measuring the retention of a tracer dose of radioactive iodine, Chai, Amin and Reineke(5) found consistent differences in thyroid hormone output rate between the inbred mouse strains A/Jax, BALB/c, C₅₇BR/cd and C₅₇BL/6.

The present knowledge of the action of antithyroid drugs might imply that the strains which normally have the highest thyroid hormone output rate are also most affected by administration of an antithyroid drug and thus will show the strongest histological reaction. To test this hypothesis, in the same strains as in the above mentioned experiments of Wilson, I¹³¹ uptake and retention were measured in the present experiments.

Materials and methods. Male mice, 60 to 90 days of age, of the following strains were used: BUB/Wi, BUC/Wi, C₃H/AnWi, C₃H/StWi, C₅₇BL/Fn and YBR/HeWi. In the YBR/HeWi strain only the yellow mice, heterozygous for the A^y gene were used. The mice were raised on Purina Laboratory Chow, and kept at constant temperature (78°F). Fifteen days before experiment, 12 mice of each strain were transferred to a basal diet consisting of 20% Crisco, 30% Vitamin test casein (G.B.I.), 29% Argo corn starch, 8% powdered brewer's yeast (G.B.I.), 7% salt mixture U.S.P. XII #2 (G.B.I.), 4% Mazola corn oil and 2% cod liver oil (Squibb). In each experiment the mice were injected intraperitoneally with ca. 4 μ c of a carrier-free NaI¹³¹ solution. A standard solution was prepared containing 1/50 of the dose injected into a mouse. Each mouse was counted externally at 10, 16, and 22 hours after injection, and on the following days once or twice a day. For this *in vivo* counting, the thyroid region of the mouse was held in front of the slit-formed aperture in the lead shielding of

the counter.† In order to compensate for the extrathyroidal background and to enable the comparison between *in vivo* counts and counts of the standard solution, the geometric conversion ration, R, was determined by the method of Fish, Carlin and Hickey(6) for each strain of mice. The fractional uptake, F, is calculated as follows:

$$F = \frac{\text{Counts/min. of mouse } in \text{ vivo}}{\text{Counts/min. of standard solution}} \times R \times 0.02$$

The logarithm of the fractional uptake is plotted against the time after dose administration in hours. The point at which output of I¹³¹ surpasses uptake of I¹³¹ is referred to as maximum uptake. This peak is usually reached by 22 hours after injection. After the peak a straight regression line is fitted by the method of least squares. The regression coefficient measures the slope of the retention line and thus represents the rate of loss of I¹³¹ from the thyroid. Since the organic binding of iodine is a rapid process and since eventually most, if not all, of an injected tracerdose I¹³¹ is incorporated into thyroid hormone, this slope is considered to reflect the secretion rate of I¹³¹ labeled hormone.

Results. The maximum uptake and the regression coefficient of 7 identical experiments are averaged for each strain and summarized in Table I.§ Maximum uptake for BUB/Wi, BUC/Wi and C₃H/Wi mice is around 19% (18.8%, 20.4% and 19.2% respectively), whereas the C₅₇BL/Fn and YBR/HeWi strains have a lower maximum uptake (12.3% and 13.0% respectively). The retention line is steepest in the C₅₇BL/Fn mice (-0.0039) and thus this strain is concluded to have the fastest output of thyroid hormone. The BUB/Wi strain has the second highest rate of output (-0.0025), followed by the C₃H/Wi strain (-0.0023), the YBR/HeWi strain (-0.0021) and the BUC/

† Grateful acknowledgement is made to Dr. F. C. Hickey, O.P., for lending this part of the counting equipment and for his help in this investigation. For description, see reference (6).

§ C₃H/Wi refers to the 2 sublines C₃H/AnWi and C₃H/StWi, the results of which have been combined, because they were in good agreement.

TABLE I. Average Uptake and Retention of Radioiodine in Different Mouse Strains.

Strain	No. of experiments*	Avg maximum uptake† (%)	Avg regression coefficient†	Reaction to 0.5% sulfadiazine (Wilson, 3)
C ₅₇ BL/Fn	2	12.3 ± 1.8	-0.0039 ± .00037	+
BUB/Wi	7	18.8 ± 2.3	-0.0025 ± .00008	++++
C ₃ H/Wi	3	19.2 ± 1.4	-0.0023 ± .00010	++++
YBR/HeWi	2	13.0 ± 2.7	-0.0021 ± .00042	+++
BUC/Wi	3½	20.4 ± 3.6	-0.0019 ± .00014	++

* Each experiment represents 12 mice/strain.

† Values are given with their stand. error of the mean.

Wi strain (-0.0019). In spite of considerable variation between experiments, this order is generally observed in each individual experiment.¶ Analysis of variance showed that the variance attributable to strains is highly significant ($P < 0.005$).

Discussion. The order in which the strains in the present experiments have been found to rank in their normal thyroid hormone output rate does not parallel the order of degree of histological reaction after sulfadiazine feeding (Wilson, 3). This does not support the hypothesis that the strain which normally has the highest thyroid hormone output rate would be most affected after administration of sulfadiazine, and thus show the strongest histological reaction. Degree of reaction following sulfadiazine treatment is thus not directly dependent upon the normal rate of thyroid hormone output. The order in which the strains in the present experiments can be arranged according to their maximum uptake also does not coincide with the order of the reaction to sulfadiazine. The maximum uptake is an index for the function of the thyroid in so far as it is dependent upon the iodide concentrating ability of the thyroid, but the value found for the maximum uptake is also influenced by rate of output of iodine by the thyroid. In the present experiments no inverse relationship was found between the order of output rate and maximum uptake of the strains. Therefore, the differences in maximum uptake among the strains reflect true differences in rate of I^{131} uptake.

It was expected that differences in thyroid function between strains could be explained by the assumption that in one mouse strain

the pituitary-thyroid axis operates at a higher level than in another strain. D'Angelo (7) and coworkers found that the pituitary-thyroid axis functions at a much higher level in the rat than in the guinea pig. In the rat the thyrotropin content of blood and adenohypophysis is high, the thyroid takes up a large percentage of injected I^{131} and secretes I^{131} relatively fast; whereas in the guinea pig thyrotropin content is low and rate of I^{131} uptake and output is slow. If in one mouse strain the pituitary-thyroid axis operates at a higher level than in another, the strain with the highest thyrotropin content should have the highest uptake of I^{131} , the largest output rate, and the most hypertrophied thyroid in case of intervention with the normal thyroxine production by sulfadiazine. This was not found in the present experiments. The thyrotropin content of the pituitary is not known, but a strain with a high maximum uptake did not necessarily have a fast output rate, nor a strong reaction to sulfadiazine. This lack of correlation between the 3 indices of thyroid function measured leads to the conclusion that in these strains each index reflects the pituitary-thyroid interaction in its own way, independent of each other.

Summary. I^{131} uptake and retention by the thyroid of several strains of mice was measured with an *in vivo* technic and compared with the histological reaction of the thyroid to sulfadiazine administration. The order in which the strains of mice rank in their thyroid hormone output rate was found not to parallel the order in which the strains rank in their histological reaction to sulfadiazine. Degree of reaction to sulfadiazine is thus not directly dependent upon the nor-

¶ Detailed results are listed in Ph.D. thesis of the author, University Microfilms, Ann Arbor, Mich.

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Comparative Efficacy of Intravenous and Intraperitoneal Administration of Antiserum in Passive Anaphylaxis.* (26231)

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Early workers(1) observed that the latent period in passive anaphylactic sensitization in guinea pigs varied with different routes of administration of the antiserum and was shortest following intravenous injection. For example, after intraperitoneal administration of antiserum, Doerr and Russ(2) obtained the best results by permitting 24 hours to elapse before challenging. This interval was shortened to 4 hours by injecting the antiserum intravenously. From these and similar observations, the natural deduction was made that the intravenous route of passive sensitization was more uniformly effective than other routes (3, cf4, p143). In their quantitative studies of passive anaphylaxis, Kabat and coworkers injected antiserum by the intravenous method and challenged after an interval of 48 hours. Under these conditions, they showed that about 0.03 mg of antibody nitrogen from either rabbit or guinea pig antiserum produced uniformly fatal sensitization when the animals were challenged with adequate doses of antigen (cf4, p144). However, because of its greater convenience, many contemporary investigators continue to use the intraperitoneal route for passive sensitization with the interval between injection of antiserum and challenge ranging between 24 to 48 hours(5,6,7). The

present communication deals with a quantitative comparison of the effectiveness of the intravenous and intraperitoneal routes of passive sensitization when the interval between sensitization and challenge was maintained at 48 hours.

Comparison of effectiveness of the 2 routes of sensitization was accomplished by determining the median sensitizing dose (SD_{50}) of antibody nitrogen by the 2 methods and by determining the median lethal dose (LD_{50}) of antigen in animals passively sensitized with uniform doses of antibody by both routes.

Materials and methods. Rabbit antibodies to dextran were used to produce passive anaphylaxis in guinea pigs. The antiserum was produced by injecting rabbits with saline suspensions of formalin-killed *Leuconostoc mesenteroides*[†] (Strain NRRL B-1424(8)) that had been cultured in sucrose broth. Such antisera give strong precipitin reactions to purified dextrans prepared from sucrose broth cultures(9). The serum was analyzed by the quantitative precipitin method of Heidelberg and Kendall (cf 4, p59) and found to contain 1.45 mg of antibody-N per ml.

For determination of the SD_{50} of antibody-N, 2 groups of 60 guinea pigs each, weighing between 280 and 320 g, were sensi-

*The authors gratefully acknowledge technical assistance of William J. Stein.

[†] Provided by Dr. H. M. Tsuchiya, U.S.D.A.

TABLE I. Comparison of Sensitizing Dose of Dextran Antibody after Intraperitoneal and Intravenous Administration.

Route of sensitization	Mortality ratios					
	Sensitizing dose in μg antibody-N					
	2.2	3.6	5.1	7.3	11	15
Intraper.	0/10	5/10	2/10	7/10	9/10	10/10
Intrav.	0/10	2/10	3/10	6/10	6/10	9/10

Guinea pigs challenged with 1 mg dextran intrav. 48 hr after sensitizing dose.

tized with dosage levels of antiserum ranging from 2.2 to 15 γ of antibody-N. One group was sensitized by the intraperitoneal route and the second group by the intravenous route. Forty-eight hours later, all the animals were challenged, intravenously, with 1 mg of dextran.[†]

For determination of LD_{50} of dextran, 2 groups of 36 guinea pigs each were sensitized with 116 γ of antibody-N from the same antiserum. One group was sensitized by the intraperitoneal route and the second group by the intravenous route. Forty-eight hours later animals in both groups were challenged, intravenously, with dosage levels ranging from 10 to 34 γ of dextran.

Data collected for determinations of the median sensitizing and median lethal doses were used to construct dosage-response curves by the system of computation developed by Bliss and described earlier(10). SD_{50} and LD_{50} were then computed from the equations of the respective dosage-response curves.

Results. Data showing mortality ratios for the 2 groups of guinea pigs that had been passively sensitized with different doses of antidextran serum and challenged, intravenously, 48 hours later with 1 mg of dextran are recorded in Table I. With the exception of 2 animals, one in each group, all of the survivors showed slight to severe symptoms of anaphylaxis with complete recovery within a few hours. The fatal reactions terminated in death within 2 to 8 minutes. The calculated SD_{50} of antibody-N was $5.5 \pm 0.6 \gamma$ for the intraperitoneal route of sensitization and $7.1 \pm 0.9 \gamma$ for the intravenous route. The difference was not statistically significant

($P = 0.10$). The SD_{50} of antibody-N, calculated from the combined data, was $6.2 \pm 0.6 \gamma$.

Table II records data collected for determination of the LD_{50} of dextran in 2 groups of guinea pigs passively sensitized 48 hours previously with antidextran serum containing 116 γ of antibody-N per animal. All of the survivors showed slight to severe symptoms of anaphylaxis and the fatal reactions terminated in death within $2\frac{1}{2}$ to 5 minutes. The calculated LD_{50} of dextran for animals sensitized by the intraperitoneal route was $18.4 \pm 2.1 \gamma$, and for animals sensitized by the intravenous route the LD_{50} was $18.6 \pm 1.3 \gamma$.

Discussion. Our results show that the intraperitoneal and intravenous routes of injection of antiserum were equally effective in development of passive anaphylactic sensitization when the animals were challenged after an incubation period of 48 hours. Accordingly, the more convenient intraperitoneal method of passive sensitization described here may safely be used in preparation of uniformly sensitized guinea pigs for quantitative assay of antigens.

To compare our results with those of earlier workers, the data of Kabat and Landow (cf4, p 144) were treated by the Bliss statistical method for calculation of SD_{50} of antibody-N. The calculated SD_{50} was $13 \pm 2 \gamma$ antibody-N for the antiovalbumin rabbit serum and $13 \pm 3 \gamma$ antibody-N for type III antipneumococcal rabbit antibody. These results are somewhat higher than the value of $6.2 \pm 0.6 \gamma$ antibody-N for the antidextran rabbit serum found in this report, but are probably within the limits to be ex-

TABLE II. Comparison of Lethal Doses of Dextran in Guinea Pigs Passively Sensitized by Intraperitoneal and Intravenous Routes.

Route of sensitization	Mortality ratios			
	Shock dose in μg			
	10	15	23	34
Intraper.	1/8	4/10	5/10	8/8
Intrav.	0/8	2/10	8/10	8/8

Animals were sensitized with dextran antiserum containing 116 γ antibody-N and challenged intrav. 48 hr later.

[†] Provided by Dr. Allene Jeanes, U.S.D.A.

pected from different laboratories using animals from different colonies.

Summary. The intraperitoneal and intravenous routes of injection of antiserum were equally effective in development of passive anaphylactic sensitization when the guinea pigs were challenged after an incubation period of 48 hours.

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Protective Influence of Glucose Cycloacetoacetate on Glutathione Depletion in Dietary Liver Necrosis.* (26232)

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Leaf and Neuberger(1) observed that a low protein diet containing 100 mg of dry yeast per day per rat as the sole source of protein, produced an instantaneous fall in liver GSH, which after 14 days reached a constant level corresponding to 20 to 30% of the initial values. Further, this depletion of GSH was prevented by addition of cystine to the same diet. Similar results were also obtained by Lindon and Work(2) who showed that a necrogenic diet containing 18% Baker's yeast as protein source lowered liver GSH level in rats to one-fourth of its normal value in 13 days.

Recently, Nath and Behki(3) have shown that sodium salt of glucose cycloacetoacetate (GCA), a condensation product of glucose and acetoacetic ester(4) can prevent depletion of blood GSH in acetoacetate induced diabetes. The authors(5) have also observed the GSH-sparing effect of GCA in blood of scorbutic guinea pigs. It was, therefore, thought desirable to study the in-

fluence of GCA and its hydrolyzed product on depletion of GSH during feeding of necrogenic diet to rats.

Since the level of ascorbic acid in rats has also been found to be significantly depressed during development of liver necrosis (2) and since GCA has been shown to be the precursor of Vit. C in germinating legumes and in rats by Nath *et al.*(6,7), the influence of GCA and its hydrolysis product on depletion of liver ascorbic acid has also been studied. Further, in view of the protective influence of Vit. B₁₂ in CCl₄ liver injury in Vit. E deficient rats(8), analogous data comprising the influence of Vit. B₁₂ have been collected.

Experimental. Animals and diet: Thirty-five weanling male albino rats weighing 40-60 g were divided into 5 groups as follows: Animals in Group I were fed normal stock diet consisting of casein 12%, cane sugar 36%, arrowroot 40%, groundnut oil 6.4%, codliver oil 1.6%, salt mixture 3.5%(9), L. cystine 0.3%, choline chloride 0.2%. The following vitamins were added per kg of the

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TABLE I. Protective Influence of Vit. B₁₂ and Hydrolyzed GCA on Depletion of GSH in Blood and Liver in Rats during 35 Days of Feeding Necrogenic Diet.

Group and treatment	Blood GSH		Liver GSH	
	mg % $\pm$ S.D.	% of control	mg % $\pm$ S.D.	% of control
I Normal stock diet (control)	33.0 $\pm$ 5.6	—	135.3 $\pm$ 17.6	—
II Necrogenic diet	16.9 $\pm$ 2.0	51.2	88.6 $\pm$ 26.4	65.4
III Necrogenic diet + .3% crystalline GCA in diet	17.9 $\pm$ 3.5	54.2	110.1 $\pm$ 15.2	81.3
IV Necrogenic diet + 30 mg/kg of hydrolyzed GCA inj. on alternate days	27.2 $\pm$ 4.8	82.4	120.5 $\pm$ 11.2	89.0
V Necrogenic diet + 200 μ g/kg of vit. B ₁₂ inj. on alternate days	25.4 $\pm$ 4.5	76.9	127.2 $\pm$ 12.6	94.0

above diet : thiamine hydrochloride 5 mg, riboflavin 5 mg, pyridoxin 5 mg, Ca-d. pantothenate 25 mg, inositol 50 mg, biotin 0.1 mg, folic acid 1 mg, Vit. B₁₂ 25 μ g, α -tocopheryl acetate 25 mg, Vit. K 5 mg. Animals in Group II were fed the necrogenic diet as employed by Lindon and Work(2) modified as follows : Baker's yeast 6%, cane sugar 36.5%, arrowroot 46%, codliver oil 1.0%, groundnut oil 6.4% and salt mixture 3.5%. Animals of Groups III, IV and V were kept on the same necrogenic diet as in Group II animals. Group III animals were given, along with their diet, crystalline GCA at 0.3% level. Group IV animals were injected subcutaneously, on alternate days, with 30 mg equivalent hydrolyzed product of GCA/kg body wt. Group V animals were injected subcutaneously with 200 μ g/kg body wt. of Vit. B₁₂ (B-Douza, Lab. Grimault, Paris). All the animals were fed the diets and water *ad libitum*.

Analytical methods. GCA was prepared according to the method of West(4). Hydrolyzed product of GCA was prepared as follows: In a 50 cc conical flask 2 g of crystalline GCA was hydrolyzed with 10 cc 2N HCl over a boiling water bath for 20 minutes. On cooling, the contents of the flask were neutralized and extracted 3 times with ether discarding the upper ethereal layer. Air was bubbled through the aqueous extract until free from ether. Its final pH was adjusted to 7.2 with 0.1 N NaOH.

The rats were killed by a blow on the head and immediately after veni-puncture, the blood was collected in an oxalated container

and its glutathione content determined by the procedure of Woodward and Fry(10). After taking blood, a few pieces of liver (about 1 g) were cut out, dried on filter paper and dropped quickly into a weighed beaker containing about 10 cc of 2% sulphosalicylic acid solution. Ascorbic acid was determined by titration with indophenol and GSH was estimated according to a method of Woodward and Fry(10) modified by Lindon and Work(2).

Results and discussion. During 5 weeks of feeding necrogenic diet, liver and blood GSH levels were found to be reduced to 65% and 51% respectively of the average values for normal animals (Table I). The fall in level of blood GSH can be compared to that recorded by Lindon and Work(2) who observed a fall in blood GSH to 68% of initial value in 13 days. However, the reduction in liver GSH to 65% of average normal value as recorded by us, deviates considerably from the results of Leaf and Neuberger (1) and Lindon and Work(2), who recorded a reduction of GSH levels to 20-30% of controls. The disparity in our results can be ascribed mainly to 2 factors, *viz.* the quality of yeast employed as a source of protein in the diet and the delay in feeding necrogenic diet during the weanling stage. The importance of these 2 factors in liver necrosis has been demonstrated by Naftalin(11).

Table I shows that levels of liver GSH in rats (fed on necrogenic diet only) were lowered to 65.4% of normal controls whereas in the animals injected with Vit. B₁₂ and hydrolyzed GCA, corresponding values of GSH re-

TABLE II. Sparing of Ascorbic Acid by Hydrolyzed GCA and GCA in Rats Fed on Necrogenic Diet for 35 Days.

Group and treatment	Liver ascorbic acid	
	mg % $\pm$ S.D.	% of control
I Normal stock diet	24.7 $\pm$ 3.8	—
II Necrogenic diet	15.7 $\pm$ 4.3	63.5
III Necrogenic diet + 0.3% crystalline GCA in diet	22.1 $\pm$ 1.9	89.4
IV Necrogenic diet + 30 mg/kg body wt of hydrolyzed GCA inj. on alternate days	23.0 $\pm$ 2.6	93.1
V Necrogenic diet + 200 μ g of vit. B ₁₂ inj. on alternate days	20.9 $\pm$ 2.7	84.6

duction were 94% and 89% of controls respectively, showing a marked GSH-sparing influence of hydrolyzed GCA.

Crystalline GCA administered orally has been found to be less active than the hydrolyzed product, probably because of poor absorption of GCA through gastrointestinal tract. Nath and Saikia(12) have observed that methionine content of the liver in rats injected with hydrolyzed GCA is considerably higher than that of animals fed on high protein diet during development of experimental atherosclerosis. GSH-sparing influence of hydrolyzed product of GCA may, as such, be explained broadly by virtue of its ability to spare methionine, which has been shown by Leaf and Neuberger(1) to check depletion if liver GSH.

During feeding of necrogenic diet level of liver ascorbic acid was also lowered to 63.5% of normal control animals (Table II). A similar observation was also recorded by Lindon and Work(2). It was interesting to find that both GCA and its hydrolyzed product checked depletion of ascorbic acid considerably. Vit. B₁₂ was inferior in this respect. Sparing of liver ascorbic acid by GCA may possibly be explained by the role of GCA as

precursor in biosynthesis of Vit. C as shown by Nath and coworkers(6,7) in germinating legumes and in rats.

Summary. The influence of glucose cycloacetoacetate (GCA), its hydrolysis product and Vit. B₁₂ on depletion of blood and liver glutathione and liver ascorbic acid in rats during 35 days of feeding necrogenic diet containing 6% baker's yeast as the sole protein source, was studied. GSH level in blood and liver in animals receiving necrogenic diet was reduced to 51% and 65% of normal controls, whereas in Vit. B₁₂ injected animals corresponding values were 89% and 94% and in animals injected with hydrolyzed GCA respective values were 82% and 99%. Liver ascorbic acid of the animals fed on necrogenic diet was reduced to 63% of controls, while that in the animals injected with Vit. B₁₂ and with hydrolyzed GCA it was recorded to be 93% and 84% of controls respectively.

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Effects of Thiouracil on Turnover of Thyroidal I^{131} and Thyroxine Secretion Rate Measurement. (26233)

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Technics which involve simultaneous administration of thyroxine and thiouracil for determination of thyroxine secretion rate (1,2) have long been used, and require the sacrifice of the animals. Use of radioiodine has provided a much improved technic for determination of thyroxine secretion rate, and eliminates the need to sacrifice the animals. Reineke and Singh(3) devised a thyroxine-substitution method which permits determination of thyroxine secretion rate of individual rats based on inhibition of thyroidal I^{131} -output by progressively increasing doses of thyroxine. Recently, Pipes *et al.*(4,5,6) described a modification of the thyroxine substitution method in which thiouracil is used to prevent recycling of I^{131} from the metabolized thyroid hormone, and to enhance the sensitivity and accuracy of the assay. By this means they measured thyroxine secretion rate in cattle and chickens. The present work was designed to compare thyroxine secretion rate measured with thiouracil or without thiouracil, and to clarify the effects of thiouracil on thyroidal I^{131} -turnover in the chicken.

Methods. Four-week-old cockerels weighing 210 g, 12-month-old cocks weighing 1930 g, hens weighing 1730 g, and 24-month-old hens weighing 1680 g, all of White Leghorn stock, were used. Immature chickens were reared in an electric brooder kept at 80°F, and mature chickens were placed in battery cages at room temperature of about 70°F. The birds were maintained on the standard ration used at Nat. Inst. of Agric. Sciences. Thiouracil (Nutritional Biochemicals Corp., Cleveland) was given at the ratio of 0.1% in the ration. This amount of thiouracil blocks the synthesis of thyroid hormone by the thyroid in chickens(7). Immature and mature chickens were injected subcutaneously with 15 and 30 μ c of carrier-free I^{131} as NaI, respectively. The γ -radiation from

the thyroid region of immature and mature chickens was counted with a scintillation counter (Inst. of Physics and Chemistry, Ltd., Tokyo) with lead collimators with openings of 3 cm and 7 cm, respectively. The unanesthetized immature and mature chickens were placed in an immobilization apparatus during measurement of radioactivity at distances of 7 cm and 20 cm between the crystal in the counter and the surface skin of the thyroid region, respectively. Counts were corrected for background, and for physical decay at the measurement of release of I^{131} . Immature chickens were divided into 3 groups: one group maintained on normal diet, a second group on 0.1% thiouracil diet for 2 days before I^{131} -injection, and a third group on 0.1% thiouracil diet for 7 days before I^{131} -injection. Thiouracil feeding was continued after I^{131} -injection. Thyroidal uptake of injected I^{131} in percent was measured at 6 and 24 hr after injection. The apparent release rate of I^{131} from the thyroid was expressed as percentage loss of thyroidal I^{131} per day and calculated using following equation: Release rate (%) = $100 - (48 \text{ hr uptake \%} / 24 \text{ hr uptake \%}) \times 100$. Thyroidal I^{131} -uptake was measured *in vivo* using I^{131} standards in phantoms. These coincided with scintillation spectra of the thyroids of the chickens. In mature chickens thyroidal I^{131} uptake was measured at 6 and 24 hr after injection of I^{131} ; the apparent thyroidal I^{131} -release rate during the period from 48 hr to 120 hr was calculated from the regression of points of the counts plotted semi-logarithmically *vs.* time, and expressed as percentage loss of thyroidal I^{131} per day. Thiouracil feeding was started 120 hr after injection of I^{131} and thyroidal I^{131} -release rate for the first 24 hr after the start of thiouracil feeding was measured. For plasma studies, blood was taken from wing vein of five 12-month-old hens receiving 100 μ c I^{131} ,

just before the start of thiouracil feeding at 96 hr after injection of I^{131} , at 24 and 48 hr after the start of thiouracil feeding. Total plasma I^{131} and TCA-precipitable I^{131} (PBI ^{131}) were measured with a well-crystal scintillation counter (Nuclear - Chicago Corp.) From both values inorganic I^{131} (iodide ^{131}) was obtained by difference(8). To measure the extent of thyroidal recycling of I^{131} from metabolized thyroid hormone, normal diet-fed immature and mature chickens and thiouracil-fed immature chickens were injected intravenously with 0.2 μ g L-thyroxine labelled with 8 μ c I^{131} (Abbott Labs., Oak Ridge). Rate of recycling I^{131} was measured at 6 and 24 hr after injection of radiothyroxine in immature chickens, and at 24 hr in mature chickens, because the peak recycle value of immature and mature chickens was observed around 6 and 24 hr after injection, respectively. In another group of immature chickens thiouracil was given 48 hr after injection of I^{131} , and thyroidal I^{131} -release rate was expressed as percentage loss of thyroidal I^{131} for the first 24 hr after start of thiouracil feeding. For measurements of thyroxine secretion rate, immature chickens were divided into normal diet-fed and thiouracil-fed groups, the latter being pre-fed thiouracil before the thyroxine substitution as follows: for 24, 48 or 120 hr, and thiouracil was continued after the start of the substitution, which was started at 4 weeks of age. I^{131} was injected subcutaneously 72 hr before the substitution in each case. Thyroid count and body weight were measured daily. Daily graded increasing levels of L-thyroxine (Nutritional Biochemicals Corp., Cleveland), dissolved in an alkaline solution, of 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16 and 18 μ g per 100 g body weight were injected. In most cases of thiouracil-fed chicks, some of the described doses were skipped. Thyroxine secretion rate was estimated as a given dose of thyroxine which resulted in coming to an equilibrium or to a little rise in comparison with the preceding count as indicated by the external thyroid count. When a thyroxine dose producing

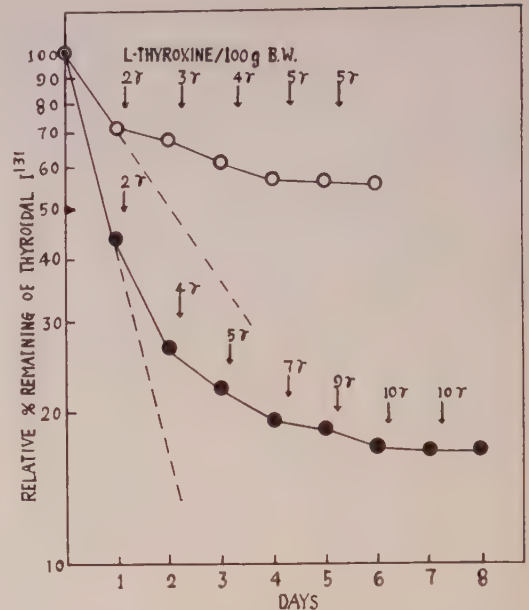


FIG. 1. Typical procedures for determination of thyroxine secretion rates in 4- to 5-wk-old cockerels. $\bigcirc$ — $\bigcirc$ Normal diet-fed bird. $\bullet$ — $\bullet$ 0.1% thiouracil-fed bird.

equilibrium or a rise in external thyroid radioactivity of I^{131} was found, the same dose of L-thyroxine was injected repeatedly (Fig. 1). Estimated true release rate of I^{131} from the thyroid(T) was calculated from the apparent release rate of I^{131} from the thyroid(A) and rate of peak recycling of I^{131} from metabolized thyroid hormone(R), using an equation of $T(\%) = A(\%) \times 100 / 100 - R(\%)$.

Results. The effect of thiouracil on turnover of I^{131} by the thyroid in 4-week-old cockerels is summarized in Table I. Maximum percent uptake of I^{131} by the thyroid was observed 6 hr after I^{131} injection. Administration of thiouracil had a statistically significant body growth depressant effect, and it significantly reduced uptake and recycling of I^{131} by the thyroid in immature chickens. Rate of recycling of I^{131} derived from the metabolized thyroid hormone, measured by accumulation of I^{131} from I^{131} -labelled L-thyroxine, was always a little less than rate of uptake of I^{131} by the thyroid. Both the uptake and the recycling of thyroidal I^{131} significantly decreased with

TABLE I. Effect of Thiouracil Feeding on Turnover of I¹³¹ by Thyroid of 4-Week-Old Cockerels.

	Normal diet	0.1% thiouracil in diet	
		Fed for 2 days before inj.	Fed for 7 days before inj.
Body wt at I ¹³¹ inj., g	208 ± 3 † (90)	190 ± 4 * (20)	189 ± 3 * (20)
6 hr uptake, %	43.58 ± .73 (80)	31.10 ± 4.40* (10)	8.79 ± .68* (10)
24 " " , %	34.65 ± .80 (80)	20.08 ± 2.97* (10)	2.92 ± .66* (10)
6 " recycle, ‡ %	37.71 ± 1.30 (10)	23.48 ± 2.57* (10)	—
24 " " , %	29.07 ± 1.21 (10)	9.54 ± 3.05* (10)	2.24 ± .96* (10)
Apparent release rate, § %	27.54 ± 1.29 (40)	67.03 ± 4.24* (10)	77.96 ± 1.81* (10)

* Difference from value of birds maintained on normal diet statistically significant at 1% level.

† Mean and stand. error.

‡ Expressed as % uptake of liberated I¹³¹ by thyroid 6 hr after radiothyroxine inj.

§ " " " " loss of thyroidal I¹³¹/day between 24 and 48 hr after inj.

Figures in parentheses = No. of birds used.

length of the period of thiouracil feeding. Thiouracil feeding caused a significant increase in release rate of I¹³¹ from the thyroid. This effect of thiouracil became progressively more pronounced as length of time of thiouracil feeding before I¹³¹ dosage was increased. Thyroxine secretion rate measured by substitution with L-thyroxine in 4 to 5-week-old cockerels is shown in Table II. Thyroxine secretion rate expressed either in µg/100 g body weight or per bird significantly increased in thiouracil-fed chickens compared with that in normal diet-fed chickens, and further increased as length of period of thiouracil feeding was increased. Thiouracil feeding also significantly increased release rate of thyroid I¹³¹ in mature cocks and hens (Table III). The maximum up-

take of I¹³¹ by the thyroid of mature chickens occurred around 24 hr after injection of I¹³¹. The percent recycling of I¹³¹ from metabolized thyroid hormone was somewhat lower than percent uptake of I¹³¹ by the thyroid. Both uptake and release rate of thyroidal I¹³¹ were lower in cocks and hens than in immature chickens. The observed release rate of thyroidal I¹³¹ during the first 24 hr following thiouracil feeding was much higher than the estimated true release rate of I¹³¹ calculated from the apparent release rate and rate of peak recycling. Such an increase in release rate of thyroidal I¹³¹ is not only due to blocking the recycling of I¹³¹ by the thyroid but also to increase of the release rate of I¹³¹ from the thyroid by thiouracil. Plasma iodide¹³¹ concentration increased 2-3

TABLE II. Effect of Thiouracil Feeding on Thyroxine Secretion Rate Measured by Substitution with L-Thyroxine in 4- to 5-Week-Old Cockerels.

	Normal diet	0.1% thiouracil in diet		
		Fed for 24 hr before substitution	Fed for 48 hr before substitution	Fed for 120 hr before substitution
No. of birds	40	20	15	10
Body wt at estimation, g	311 ± 6*	309 ± 5	285 ± 10†	292 ± 4
Thyroxine secretion rate, µg/100 g body wt/day	4.61 ± .19	7.00 ± .53‡	7.97 ± .71‡	11.67 ± 1.18‡
Thyroxine secretion rate, µg/bird/day	14.40 ± .70	21.62 ± 1.55‡	23.00 ± 1.82‡	33.89 ± 3.19‡

* Mean and stand. error.

† Difference from value of birds maintained on normal diet statistically significant at 5% level.

‡ Difference from value of birds maintained on normal diet statistically significant at 1% level.

TABLE III. Effect of Age on Uptake, Recycling, and Release of I^{131} by the Thyroid of the Chicken.

	Body wt, g	6 hr I^{131} uptake	24 hr I^{131} uptake	24 hr I^{131} recycling	I^{131} release rate/day	Estimated true re- lease rate/day	I^{131} release rate 1st 24 hr after 0.1% thiouracil feeding
		%					
4-wk-old cock- erel	208	43.58 $\pm$.73† (80)	34.65 $\pm$.81 (80)	29.07 $\pm$ 1.21 (10)	27.54 $\pm$ 1.29 (40)	44.21	57.71 $\pm$ 2.78 (40)
12-mo-old cock	1928	17.06 $\pm$ 1.60 (10)	20.80 $\pm$ 2.92 (10)	17.84 $\pm$ 3.36 (10)	5.63 $\pm$.93* (10)	6.85	12.57 $\pm$ 3.27 (10)
12-mo-old hen	1726	13.28 $\pm$ 1.11 (10)	16.40 $\pm$ 1.63 (10)	13.99 $\pm$ 1.91 (10)	3.80 $\pm$.50* (10)	4.42	7.87 $\pm$.97 (10)
24-mo-old hen	1676	10.94 $\pm$ 1.75 (10)	11.47 $\pm$ 1.90 (10)	10.88 $\pm$ 1.27 (10)	2.60 $\pm$.60* (10)	2.92	—

* Calculated from regression of plots for 4 days.
Figures in parentheses = No. of birds used.

† Mean and stand. error.

times 24 hr after the start of thiouracil feeding as compared with the level before the treatment, while plasma PBI^{131} level remained at pre-treatment level until 48 hr after the start of treatment.

Discussion. This study as well as that of Pipes *et al.* (5,6) demonstrates that thiouracil feeding significantly augments release rate of I^{131} from the thyroid. Pipes *et al.* (5,6) attributed this increase in the rate to blocking of reutilization of I^{131} derived from metabolized thyroid hormone, and stated that the rate observed in thiouracil-treated birds was a true rate of release. However, our work shows that this increase is a composite of the blocking of reutilization of hormonal I^{131} as well as increase of release rate of I^{131} itself. Furthermore, the rate of loss is accelerated as length of the period of thiouracil feeding increases. Recently, Kobayashi and Gorman (9) reported that thiouracil blocked hormone synthesis at the monoiodotyrosine stage in the young chicken. Administration of thiouracil may result in prompt release of iodide¹³¹ from the thyroid, suggested by the present work. Rosenberg *et al.* (8) reported that injection of TSH resulted in a prompt release not only of PBI^{131} but also of iodide¹³¹ from the thyroid. Rate of recycling of I^{131} always proved a little less than rate of uptake of I^{131} by the thyroid (Table I, III). The theoretical I^{131} -release rate calculated using the equation by Brownell (10) in this experiment is too high, but it is in good agreement with the estimated true release

rate obtained from the correction for recycling of I^{131} from I^{131} -labelled thyroxine. Thyroidal uptake of I^{131} greatly decreases in thiouracil-fed birds (9,11) and decreases further with the advance in length of the period of thiouracil administration, as shown by the present work.

The present work demonstrates that the thyroxine secretion rate expressed either in $\mu\text{g}/100$ g body weight or per bird, increases (more than 50%) in thiouracil-fed chickens as compared with that in normal diet-fed chickens, and increases further with length of the period of thiouracil feeding. This result shows that the thyroxine substitution using thiouracil and external I^{131} counting (5,6) is not adequate for precise determination of thyroxine secretion rate, at least in the chicken; the value obtained using this procedure is high. This increase in thyroxine secretion rate measured by the thyroxine substitution by thiouracil may be due to augmenting the output of TSH from the pituitary (12). Reineke and Singh (3) reported estimated thyroxine secretion rate was about 11% higher in thiouracil-treated than in normal rats. They began the thyroxine substitution at the same time thiouracil administration started. This method of substitution may reduce TSH production by the pituitary encountered after longer periods of goitrogen treatment. Recently, Mellen and Wentworth (13) reported that the thyroxine secretion rate measured by the thyroxine substitution method with thiouracil and radioiodine was

50% higher than the value obtained from the classical goiter-prevention assay without radioiodine in the young chicken. The value derived from the classical goiter-prevention assay may be closer to normal secretion rate, because in this way thyroxine substitution usually starts on the same day as the thiouracil feeding, and the increase in TSH production by the pituitary by thiouracil is inhibited in chickens receiving the successive excess dose of thyroxine.

Summary. The effects of thiouracil on turnover of I^{131} by the thyroid in immature and mature chickens were studied. Rate of uptake of I^{131} by the thyroid was significantly reduced and release rate of I^{131} from the thyroid was significantly augmented in thiouracil-fed birds. Rate of recycling of I^{131} from the metabolized thyroid hormone measured by accumulation of I^{131} by the thyroid from I^{131} -labelled thyroxine injected was always a little less than rate of uptake of I^{131} by the thyroid. Both uptake and recycling of thyroidal I^{131} decreased with length of the period of thiouracil feeding. Rate of release of I^{131} from the thyroid increased with the lengthened time of thiouracil feeding. The thyroxine secretion rate expressed either per 100 g body weight or per bird measured by the thyroxine substitution

significantly increased in thiouracil-fed birds compared with normal diet-fed birds. It also increased with length of thiouracil feeding. Use of thiouracil is not adequate for precise determination of thyroxine secretion rate in the chicken.

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Incorporation of Tritiated Thymidine into DNA after Oral Administration.* (26234)

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Recent studies of cellular proliferation utilizing parenteral tritiated thymidine, H^3 TDR, have demonstrated widespread incorporation of this DNA precursor into proliferating cells(1,2,3). Metabolic studies with H^3 TDR in man have shown prompt utilization of this compound for DNA synthesis with degradation of approximately one-

third of the injected dose to tritiated water and excretion in the urine of only small amounts as non-volatile tritiated products (4). No information is available on the fate of ingested H^3 TDR. Such studies are needed for evaluation of the potential hazard of this material in respect to its laboratory use. In this paper autoradiographic (ARG) studies will demonstrate absorption following oral administration and subsequent incorporation

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TABLE I. H^3 Activities of Body Fluids Following H^3 Thymidine Ingestion.

		Ingested H^3 TDR, μ c	Sacrificed after	Catabolized to tritiated water, %	Urine non- volatile H^3 activity ex- cretion, %
Guinea pig	1	500	2 hr	15.8	.35
	2	100	2	16.5	.32
Rat	1	100	3	8.1	.36
	2	100	3	8.1	.23
	3	100	6	8.4	.32
	4	100	6	10.9	.41

of H^3 TDR into DNA of widespread body tissues.

Methods and materials. Adult male guinea pigs, rats, and mice were maintained on a stock diet. H^3 TDR of high specific activity, 1.9C/mM,[†] was used for all studies. Solutions of H^3 TDR were introduced into the stomachs of animals by intubation with a curved blunt needle. The animals were killed after varying times. ARG's were prepared from formalin fixed tissues by methods previously described(2,3). In some animals the penile urethras were ligated at the time of H^3 TDR ingestion to study the appearance of tritium activity in urine as well as plasma and bile. Total tritium activity and distilled volatile H^3 activity, (tritiated water) were assayed by liquid scintillation spectrometry(4). Non-volatile H^3 activity was estimated from the difference between total H^3 activity and tritiated water activity of the same sample.

In one guinea pig, tissues were obtained 2 hours after H^3 TDR ingestion along with gastric contents. These were lyophilized to remove tritiated water. Aliquots were dissolved in Hyamine and assayed for non-volatile H^3 activity using liquid scintillation counting(5).

Results. Results of H^3 counting are shown in Table I. Two hours after H^3 TDR ingestion in guinea pigs, plasma activity was predominantly tritiated water. Assuming uniform distribution in total body water, approximately 15% of ingested H^3 TDR had been catabolized to tritiated water and significant plasma non-volatile H^3 activity was not present. Tritiated water activity of the bile was similar to plasma; non-volatile H^3

was not detected. Urine from the obstructed bladders contained non-volatile H^3 activity that accounted for 0.35 and 0.32% of the ingested dose.

Autoradiographs of guinea pig tissues demonstrated extensive labeling. Heaviest label was seen in the small intestinal mucosa. Labeling in other tissues examined was scant but definite in bone marrow, spleen, lymph node, liver and kidney.

Guinea pig No. 1 received 500 μ c H^3 TDR *per os* and was sacrificed 2 hours later. Tissues and stomach contents were lyophilized and dissolved in Hyamine. Non-volatile H^3 activity of these tissues are recorded in Table II.

Similar results were obtained in 4 rats which received 100 μ c of H^3 TDR and were sacrificed at 3 and 6 hours after ingestion. Tritiated water activity was present in bile, plasma, and urine and significant but only small amounts of non-volatile H^3 activity were present in bladder urine (Table I).

To demonstrate better intranuclear labeling, a rat (200 g) and a mouse (30 g) were similarly intubated and given 100 μ c and 25 μ c H^3 TDR respectively. The animals were killed 7½ hours later. After 5 weeks

TABLE II. Non-volatile H^3 Activity of Guinea Pig Tissues 2 Hours after H^3 TDR *per os*.

	Non-volatile H^3 activity, cpm/mg dry wt
Stomach contents	442
Spleen	44
Liver	28
Bone marrow	28
Kidney	15
Ilium	11
Iliopsoas	5

[†] Schwarz BioResearch Inc., Mt. Vernon, N. Y.

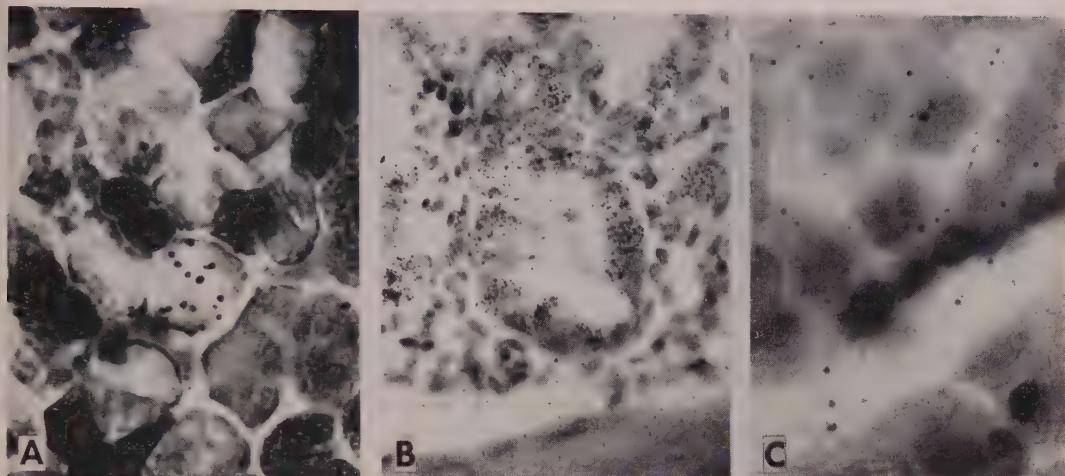


FIG. 1. ARG of rat and mouse tissues $7\frac{1}{2}$ hr after H^3 thymidine ingestion. A = mouse bone marrow; B = rat ilium; C = mouse testes.

exposure, radioautographs (Fig. 1, A, B, C), were obtained.

Discussion. The widespread labeling of proliferating body tissues after H^3 TDR ingestion strongly suggests that absorption of H^3 TDR through the intestinal mucosa occurs. The extensive labeling of gastrointestinal mucosa seen here is indistinguishable from mucosa labeled with H^3 TDR following parenteral injection(6) and after incubation of gastrointestinal mucosa *in vitro* with H^3 TDR(7).

Wilson and Wilson(8) using an *in vitro* system have demonstrated biochemically the ability of thymidine to pass intact through guinea pig intestinal mucosa.

Upon ingestion of H^3 TDR, absorption from the stomach is slow. Most of the H^3 TDR remained within the stomach contents of guinea pig I which was sacrificed 2 hours after ingestion (Table II).

The gastrointestinal mucosa was labeled most heavily in these experiments. Other tissues received only small amounts of the label. The lyophilized tissues did contain non-volatile H^3 activity (Table II). These activities are based on mg dry weight of tissues and fail to take into account the varying cellularity and extent of cell renewal populations of the tissues studied. In spite of these difficulties, the data show non-volatile H^3 was present in the spleen and other tissues with very little present in muscle. These re-

sults are similar to those seen in rat tissues after parenteral H^3 TDR(9). We know of no evidence indicating storage of H^3 TDR in tissues other than in DNA of proliferating cells.

Some H^3 TDR is catabolized to tritiated water; the site of this degradation is not known. Small amounts of readily detectable non-volatile H^3 activity are excreted in the urine after H^3 TDR ingestion. Some of this may be H^3 beta-aminoisobutyric acid as occurs after intravenous H^3 TDR injection in man(4).

Bile and plasma obtained from these animals contained tritiated water but failed to reveal the presence of any detectable non-volatile H^3 activity. The absence of non-volatile H^3 activity in bile is suggestive of the absence of any enterohepatic circulation of H^3 TDR or its degradation products. The absence of plasma non-volatile H^3 activity was expected in view of the rapid disappearance curve of injected H^3 TDR in man(4).

Hazards from H^3 TDR have been postulated(2,12) and radiation effects described(10,11,13). The labeling of spermatogonia following ingestion of H^3 TDR shown here further urges reasonable caution in handling this compound in any significant amounts.

Summary. 1. H^3 TDR was administered *via* stomach tube to rats, mice, and guinea pigs. 2. Some ingested H^3 TDR was absorbed and labeled proliferating cells of gas-

trointestinal mucosa as early as 2 hours after ingestion in guinea pigs. 3. Labeling of diverse tissues including proliferative cells of the testes was seen in rats and mice 7½ hours after ingestion. 4. Some of the H³TDR was degraded to tritiated water while small amounts of non-volatile H³ activity were excreted in the urine. 5. The significance of absorption of H³TDR through intestinal mucosa is discussed as related to potential hazard.

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Staining of Malarial Parasites by the Fluorescent Antibody Technic. (26235)

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The fluorescent antibody technic has been used successfully by a number of workers to stain a variety of organisms (1, and others). The ease with which serums may be conjugated with the isothiocyanate derivative of fluoerscein and the stability of the labeled preparation has rapidly advanced the method from one of a purely research tool to one which is developing into a diganostic instrument as well. Our study was made to bring this useful and proven procedure to the study of malariology where its research and diagnostic potential appear promising. The following experiments were undertaken: (1) to determine whether natural infections produce antibodies which could be tagged with the dye to detect the organisms in the host; (2) whether antibodies could be produced in rabbits by a series of injections with malarial organisms and (3) whether one stage of the parasite, *e.g.*, the sporozoite which develops in the mosquito, could be used to produce antibodies which would react

with the erythrocytic stage in the vertebrate host.

Materials and methods. Serum was collected from 2 *Macaca mulatta* monkeys which had been inoculated originally with blood containing about 2.0×10^8 parasites of *P. cynomolgi bastianellii*. Each of these animals developed a high parasitemia (average 2.0×10^5 parasites/mm³ of blood). They apparently had a spontaneous recovery from the disease. About 7 months later both animals were reinoculated with 2.0×10^8 parasites and again developed a fairly high parasitemia (about 5.0×10^4 parasites/mm³). After approximately 6 weeks the monkeys were inoculated for the third time with the usual inoculum of parasites. Neither animal developed a parasitemia. One of the animals was given a fourth inoculation one week later. No parasites were detected by blood smear examination for one week. At this time the animals were bled. The serums were pooled and the gamma globulin

fraction precipitated by 34% saturation with ammonium sulfate. Ammonium sulfate was removed by dialysis against 0.15 M saline buffered with 0.01 M phosphate, pH 7.1 until the dialysate gave a negative test for ammonia. The serum fraction thus obtained was labeled with fluorescein isothiocyanate by the technic essentially as described by Coons and Kaplan(2). Thin blood smears were made from monkeys infected with *P. cynomolgi bastianellii*; the smears were dried, fixed for 10 seconds in absolute methanol, and after drying were layered with the conjugated gamma-globulin fraction; after 30 minutes the labeled material was washed off with phosphate buffered saline, pH 7.1; the smears were then mounted using 10% buffered glycerol and examined by means of a Zeiss fluorescent microscope. For preparing antigen against blood stages of *P. cynomolgi bastianellii*, 3 to 5 ml of blood containing about 1.0×10^8 parasites per ml was taken from infected Rhesus monkeys; serum and the buffy coat were removed and red blood cells were hemolyzed by freezing and thawing 3 times. After each thawing the residue was washed with cold 0.15 M buffered saline. Finally, the residue was resuspended in the buffered saline and stored in the refrigerator at 4°C until used; 1 ml was injected intravenously once a week for 3 consecutive weeks into each of 2 white male New Zealand rabbits weighing about 2 kg. One week after last inoculation, the animals were bled, the gamma-globulin fraction was obtained and labeled as previously described. *Plasmodium cynomolgi bastianellii* infected mosquitoes were not available for determining whether or not antibodies against the sporozoite stage could be produced which would react with the erythrocytic stage. For this purpose, *Aedes aegypti* mosquitoes infected with *P. gallinaceum* were used. The antigenic material was prepared by grinding the heads and thoraces from approximately 150 infected mosquitoes in Hanks' basal salt solution; the sporozoite suspension thus obtained was strained through glass wool to remove large particulate matter; one ml of the suspension containing about 3.5×10^6 sporozoites was injected intravenously into

white rabbits as previously described. The course of injecting the parasites, collecting, and tagging the serum was carried out as in the previous study except that fresh antigenic material was made for each inoculation.

Results. Parasites of blood smears from *P. cynomolgi bastianellii* infected monkeys showed a distinct, but not a high degree of fluorescence when stained with labeled serum from experimentally infected monkeys. The morphology of the stained parasites appeared very similar to parasites stained with giemsa by conventional means. The immunological specificity of the staining was indicated by: (1) lack of staining when slides were treated with conjugated normal serum; (2) inhibition of staining by prior exposure of the parasites to unconjugated homologous immune serum; (3) failure of the conjugated anti-malarial serum to stain after being adsorbed with a suspension of the organisms and (4) failure of prior application of non-immune serum to inhibit staining. Also, the labeled immune serum failed to stain parasites of *P. gallinaceum*.

We were unable to stain the homologous parasite with the fluorescent antiserum obtained by injecting the blood phase of the parasite into rabbits.

Chicken blood smears parasitized with *P. gallinaceum* and stained with antibodies produced in rabbits with sporozoites showed a high degree of fluorescence (Fig. 1). The parasites could be seen in the red blood cells which are faintly outlined as well as is the nuclei. Specificity of staining was determined as previously described. Parasites were stained also by the indirect method of Weller and Coons(3). No staining was observed when non-immune serum was substituted for immune serum in this procedure.

The non-specific fluorescence of white blood cells was not troublesome.

Sporozoites were immobilized and agglutinated with non-labeled immune serum. Control non-immune serum appeared to have little, if any, effect on the organism. Consequently, this indicates that antibodies were produced against the sporozoites. No attempt was made to stain the sporozoites because of technical difficulties.

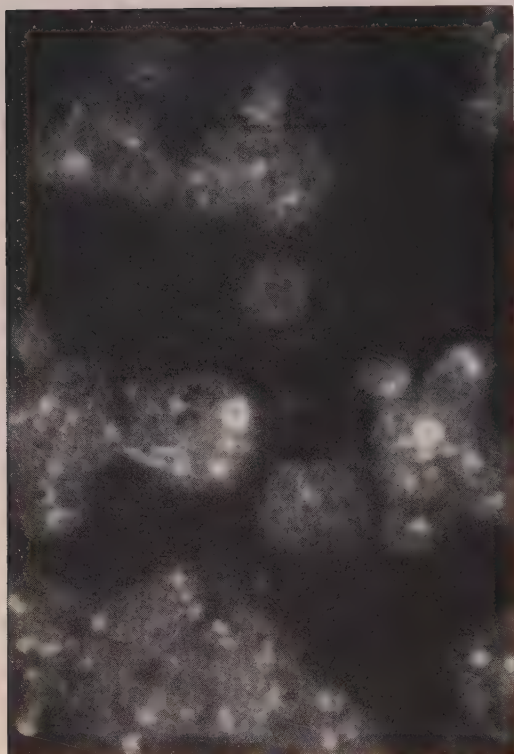


FIG. 1. Chick blood smear showing malaria parasites of *P. gallinaceum* stained with fluorescent labeled antibodies obtained from rabbits by sporozoite injections. Several non-parasitized red blood cells can be seen.

Attempts were made to stain the erythrocytic stage of *P. cynomolgi bastianellii* with the *P. gallinaceum* labeled antiserum. No staining was observed, indicating that there was some species specificity. Other species will be studied to establish the specificity of the antigens involved in the staining reaction.

Discussion. Too little is known about antigens of malarial parasites to predict much about specificity of their immunochemical reactions. One would expect that there might be some common antigens among them.

However, there probably are specific antigens which would serve to distinguish them by means of immunological methods. Common antigens between species could probably be dealt with by an adsorbent technic as was demonstrated by Goldman(4) in distinguishing between *Endamoeba histolytica* and *E. coli*.

If there is immunological specificity among the various malarial organisms our finding should offer considerable encouragement in the study of the possible relationship of the monkey to the human malarial picture. Also, the demonstration that one stage of an organism can be used to prepare an antiserum to another stage of the same organism might prove useful in investigations of many parasitic diseases of man and animals in which the parasite undergoes several stages of development. One such possibility might be elucidation of the cycle of some of these organisms which are poorly understood.

Summary. Two species of malarial parasites have been stained by the fluorescent antibody technic. *P. cynomolgi bastianellii* was stained with antibodies obtained from convalescent Rhesus monkeys experimentally infected with the parasite. The erythrocytic stage of *P. gallinaceum* was stained with antibodies produced in rabbits by injections of the sporozoite stage of the organism.

The authors thank Doctors William F. Cantrell and Don E. Eyles for their help and suggestions.

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Extra-hypothalamic Lesions Associated with Gold-Thioglucose Induced Obesity.* (26236)

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The specificity of goldthioglucose for the ventromedial nuclei and adjacent parts of the hypothalamus has been emphasized by several investigators(1,2,3). This specificity has been regarded as providing support for a theory that the ventromedial hypothalamus contains neurones that comprise the satiety portion of a "feeding center" and that these neurones are glucoreceptors. It has been postulated that gold is "dragged" into the ventromedial hypothalamic cells which are then destroyed because of their peculiar affinity for glucose. The present report deals with induction of lesions outside the ventromedial nuclear region of the hypothalamus by goldthioglucose. The amount of goldthioglucose used has already been determined to induce a massive obesity associated with lesions in the ventromedial nuclear region of the hypothalamus, and in the case of CBA mice, lesions in the region of the fornix as well(4).

Methods. The inbred C57 Black and CBA mice used in these experiments were fed Purina lab chow *ad libitum*. The C57 Black mice received an intraperitoneal injection (27 ga. needle) of 1.0-1.2 mg, or of 0.35-0.4 mg goldthioglucose† in 0.9% saline per gram body weight when they were 100 ± 10 days old. The CBA's were given 0.35-0.4 mg/g body weight, but otherwise were treated like the C57 Black mice. Autopsy was carried out 24-48 hours after injection, the brain fixed in 10% formalin or Heidenhain's Susa, and paraffin sections prepared using myelin, toluidin blue, hematoxylin and eosin, and chrome alum hematoxylin phloxine procedures. Fifteen C57 Black and 25 CBA brains were prepared as outlined and serial sections of all of these brains studied microscopically. Five additional brains from C57 Black mice that had been given 0.35 mg

goldthioglucose per gram body weight were also studied.

Results. The results of these experiments confirmed previous observations(4) regarding distribution of the ventromedial lesions in the hypothalamus. Parts of the premammillary, arcuate, and anterior hypothalamic nuclei were damaged. Almost all of the neurones of the ventromedial nucleus were destroyed if the dose of goldthioglucose was 1 or 1.2 mg/g body weight in the C57 Black, or 0.35 or 0.4 mg/g in the CBA mice. When the smaller dose was administered to C57 Black mice, the lesion failed to involve the premammillary nucleus, and damage to the arcuate, ventromedial, and anterior hypothalamic nuclei was small (Fig. 1). These lesions were not associated with weight gain.

Destruction of neurones in the visceral sensory nuclei of the vagus or nucleus solitarius, especially its commissural part, has been observed in both strains at all dose levels. Commonly some neurones of the dorsal motor nucleus of the vagus, and hypoglossal nucleus were also destroyed (Fig. 2). Less frequently the lesion involved the gracile and cuneate nuclei. All these neurone cell bodies are contiguous to the area postrema.

Near the supraoptic crest, damage from goldthioglucose occurred in the preoptic nuclei (Fig. 3).

In the sector between the subcommissural organ caudally, and the subfornical organ rostrally, goldthioglucose administration was associated with damage to neurones of the dorsal hippocampal formation (Ammon's horn and dentate gyrus), psalterium, triangular septal, and occasionally the lateral septal nuclei and epithalamus (Fig. 4, 5).

With the notable exception of lesions in the vicinity of the area postrema, neurone destruction outside the hypothalamus did not occur when 0.35 mg goldthioglucose was given to C57 Black mice. It was also to be noted that neurone damage in the preoptic

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† Furnished through the courtesy of Schering Corp., Bloomfield, N. J.

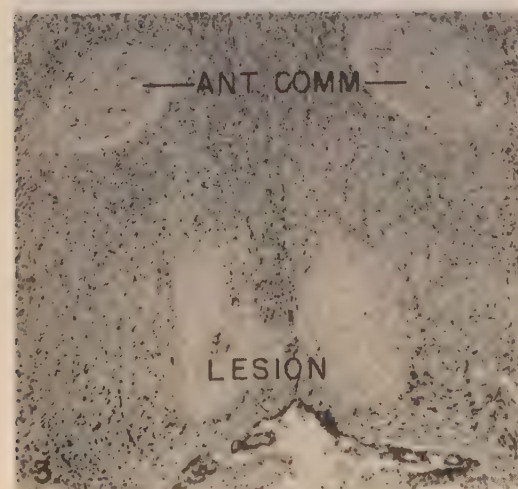
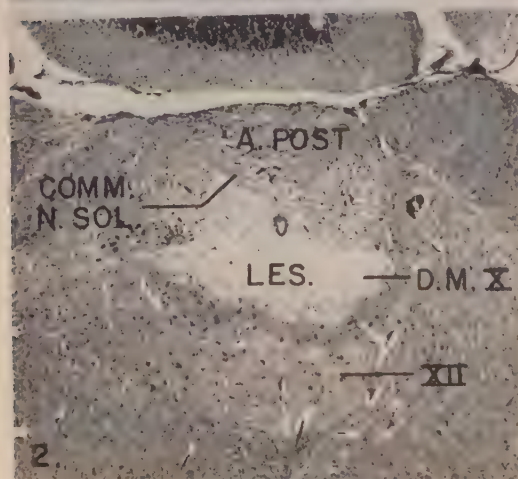
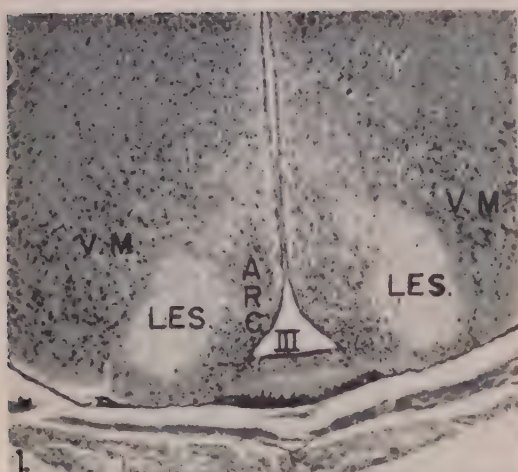


FIG. 1. Non-obese producing lesions between ventromedial (V.M.) and arcuate (ARC) nuclei of C57 BL mouse, 0.35 mg goldthioglu-
cose/g body wt.

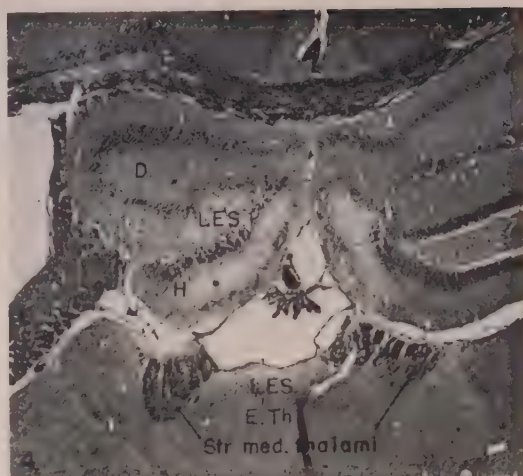


FIG. 4. Lesion in epithalamus (E.Th.) and dorsal hippocampus (D.H.) of CBA mouse, 0.35 mg goldthioglu-
cose/g body wt. Post inj. interval: 1 day. Stain: Chrome alum hematoxylin phloxine.

FIG. 5. Lesion in septum of C57 BL mouse dorsal to transverse fibers of psalterium, 1.2 mg goldthioglu-
cose/g body wt. Post inj. interval: 2 days. Stain: Weil's myelin.

nuclei was most often associated with 0.4 mg doses in CBA, and 1.2 mg of goldthioglu-
cose per gram body weight in C57 Black mice. Preoptic damage, however, was consistently

Post inj. interval: 1 day. Stain: Chrome alum hematoxylin phloxine.

FIG. 2. Lesion of dorsal motor nucleus of vagus (D.M.X.), commissural part of nucleus solitarius (Comm. N. Sol.) and some cells of hypoglossal nucleus (XII) of CBA mouse, 0.35 mg goldthioglu-
cose/g body wt. Post inj. interval: 1 day. Stain: Chrome alum hematoxylin phloxine.

FIG. 3. Lesion in preoptic region of C57 BL mouse, 1.2 mg goldthioglu-
cose/g body wt. Post inj. interval: 2 days. Stain: Toluidin blue.

noted in C57 Black mice receiving pretreatment with glucose 20 minutes before 1.0 mg goldthioglucose per gram body weight was administered.

Discussion. The results of these experiments show that administration of goldthioglucose, in amounts that have been shown to induce obesity(4), causes destruction in widely scattered areas of the brain. They are not limited to the ventromedial nucleus nor even the ventromedial region of the hypothalamus. The functions of many of the damaged nuclei, as classically defined, are rather remote from the function of regulating food intake. Damage to nuclei in the vicinity of the area postrema may occur at levels of goldthioglucose administration far below that requisite for hyperphagia and ensuing weight gain. Finally, it appears noteworthy that sites in the brain damaged by parenterally administered goldthioglucose have in common proximity to loci where the blood-brain barrier has been demonstrated to be deficient to trypan blue, to silver nitrate administered in drinking water(5), and to radioactive isotopes(6). Recent work using labeled goldthioglucose(7) demonstrated radioactivity in the hind brain as well as in the hypothalamus.

Conclusion. Goldthioglucose administration to inbred C57 Black and CBA mice, in amounts sufficient to induce hyperphagia and massive weight gain, results in lesions of nuclei adjacent to the area postrema of the medulla oblongata, to the median eminence of the hypothalamus, to the supraoptic crest between the hypothalamus and the basal telencephalon, and between the subcommissural and subfornical organs associated with the midbrain-epithalamic transition and the hippocampal formation. The hypothesis is advanced that the observed distribution of the lesions is related to deficiency of the blood-brain barrier at these sites.

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Effect of Synthetic Steroid or Phenanthrene Compounds upon Weight Gain and Feed Efficiency of Rats.* (26237)

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Previous work has shown that estrogens tend to increase rate of gain and efficiency of feed utilization in ruminant animals(1,2), but generally depress these responses in non-ruminant animals(3-5). Evidence has appeared in the literature(6,7) to indicate that modification of estrogenic compounds by oxidation reactions yields substances which are non-estrogenic and therefore have reduced inhibitory effect on non-ruminant growth, but

nevertheless retain the properties of their parent substances in inducing pituitary release of trophic factors.

Randall and Selitto(8) indicated that the compound Ro - 2 - 7239 (2 - acetyl - 7 - oxo - 1,2,4,4a,4b,5,6,7,9,10,10a - dodecahydrophenanthrene) administered subcutaneously for 7 days at a rate of .5 mg/rat/day exhibited a tendency to increase the gain of female rats. It was of interest to investigate the effect of feeding this compound at somewhat lower levels upon body weight gain and feed efficiency in female rats and in nor-

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TABLE I.

Item and animal	Diet 1, basal*	Diet 2, SC-6582	Diet 3, Ro-2-7239	S.E. of means†	L.S.D.‡
A. Effect of SC-6582 or Ro-2-7239 on wt gain and feed efficiency of adult rats					
1. Wt gain (g/28 days)					
Normal male	71.7 §	69.9 ¶	57.8 **	±6.0	11.3
Castrate "	70.0	61.8	65.6		
Normal female	57.4	52.9	52.0		
2. Feed efficiency (g gain/g feed)					
Normal male	.180	.175¶	.145**	± .012	.028
Castrate "	.176	.154	.164		
Normal female	.144	.132	.130		
B. Effect of SC-6582 or Ro-2-7239 on pituitary, thyroid and adrenal tissue/100 g of body wt of adult rats					
3. Pituitary (mg/100 g)					
Normal male	3.36§	3.02	3.08	± .28	.79
Castrate "	4.84	5.03¶	5.06¶		
Normal female	4.72	4.23¶	4.18¶		
4. Thyroid (mg/100 g)					
Normal male	4.90	5.43	5.70	± .48	1.42
Castrate "	6.04	6.10	5.96		
Normal female	6.98¶	6.82	6.30		
5. Adrenals (mg/100 g)					
Normal male	15.35	15.31	18.77	±1.36	4.41
Castrate "	18.24	17.09	20.74		
Normal female	27.13¶	28.43¶	24.60		
C. Effect of SC-6582 or Ro-2-7239 on testes or uterus/100 g of body wt of adult rats					
6. Testes (g/100 g), normal ♂	1.13§	1.28	1.36	±.11	.35
7. Uterus (g/100 g), normal ♀	.16	.12	.11	±.03	.13

* Rockland rat diet.

† Pooled stand. error of means.

‡ Least difference between means required for significance at 5% probability level.

§ Mean of 4 observations.

|| Significant at $P < .05$.¶ Significant at $P < .01$. [These symbols (||, ¶) used to compare the greater responses to the smallest response for each experimental diet.]** Significant at $P < .05$. [This symbol (**) used to compare responses to experimental diets with that to basal diet for each class of animals.]

mal and castrate male rats under the condition of controlled equalized feed intake.

Cook *et al.* (9) indicated that subcutaneous injection of approximately 5 mg or more of compound SC-6582 (17 α -methyl-17 β -hydroxy-5(10)-estren-3-one)/kg of body weight increased the rate of weight gain of cholesterol-fed cockerels. Subcutaneous injection of 1.5 mg of this compound/rat/week appeared to decrease body weight gain of castrate immature male rats. It was of interest to compare the effect of feeding this compound at somewhat lower levels to castrate male, as well as normal male and female rats under the condition of equalized feed intakes.

The present work involved feeding com-

pounds Ro-2-7239 or SC-6582 under conditions of equalized and *ad libitum* feeding to normal female and normal and castrate male albino rats. The criteria evaluated were body weight gain and feed efficiency. Weights of pituitary, thyroid, adrenal, and testes or uterus were also observed.

Materials and methods. Experiment 1: Twenty-four male and 12 female young adult Wistar rats were used. Twelve of the males were castrated under ether anesthesia 1 week prior to initiation of the feeding experiment. The animals were allotted as 4 replicates of 9 animals each in a 3 x 3 factorial design to 3 experimental diets and amount of feed offered within each replicate was limited to that of the animal consuming the least. Tap

TABLE II. Effect of SC-6582 or Ro-2-7239 on Weight Gain, Feed Efficiency, and Organ Weight of Young Rats.

Item and animal	Basal diet	Treatment means							S.E. of means*	L.S.D.†
		SC-6582			Ro-2-7239					
		.75 μ g	1.5 μ g	3.0 μ g	.75 μ g	1.5 μ g	3.0 μ g	3.0 μ g		
1. Wt gain (g)										
Normal male (25 days)	169.0	152.5	165.0	165.2	171.0	157.2	158.8		$\pm$ 6.5	19.3
" female (28 days)	126.5	106.0	115.0	120.2	113.5	115.0	126.0		$\pm$ 11.9	39.7
2. Feed efficiency (g gain/g feed)										
Normal male	.380	.365	.370	.370	.382	.359†	.359†		$\pm$.008	.02
" female	.290	.272	.263	.279	.274	.266	.290		$\pm$.029	.10
3. Pituitary (mg/100 g)										
Normal male	3.43	3.57	3.62	3.38	3.52	3.67	3.34		$\pm$.16	.47
" female	4.76	4.68	5.07	5.09	4.92	5.01	4.36		$\pm$.55	1.85
4. Thyroid (mg/100 g)										
Normal male	5.69	5.78	6.53	6.24	5.26	5.48	6.60		$\pm$.42	1.21
" female	6.68	6.44	7.12	7.34	6.60	7.16	6.62		$\pm$.61	2.05
5. Adrenals (mg/100 g)										
Normal male	14.64	15.31	15.45	17.42§	14.62	15.44	15.12		$\pm$.64	1.93
" female	26.28	27.66	28.06	26.21	31.20	29.72	25.58		$\pm$ 2.44	8.14
6. Testes (g/100 g)	.96	.93	1.04	.83	1.04	1.12	1.02		$\pm$.10	.30
7. Uterus (g/100 g)	.15	.22	.15	.12	.16	.21	.10		$\pm$.03	.11

* Stand. error of means.

† Least differences required for significance at 5% probability level.

‡ Significant at $P = < .05$.§ " " $P = < .01$.

water was given *ad libitum*. Diet 1 (basal) was a ground rat diet,† diets 2 and 3 were the basal diet with additions of 1 μ g of SC-6582‡ and Ro-2-7239,‡ respectively, per g of diet. At the end of 43 days (Replicates 1 and 4) or 37 days (Replicates 2 and 3) on experiment the animals were killed with chloroform, and pituitary, thyroid and adrenals were excised from all animals as were testes or uterus from appropriate animals.

Experiment 2: Forty-two Wistar rats (28

† Rockland rat diet, A. E. Staley Mfg. Co., Decatur, Ill.

‡ Premixes of compounds SC-6582 or Ro-2-7239 were made by mixing 0.1 g with 99.9 g cerelose. Sufficient cerelose was added to the basal diet to equal that of the premix added to experimental diets. The authors wish to thank Dr. D. L. Cook, G. D. Searle & Co., Chicago, Ill. and Dr. L. O. Randall, Hoffman-LaRoche Inc., Nutley, N. J., respectively, for generously making available supplies of compounds SC-6582 and Ro-2-7239.

males, 34-73 g, and 14 females, 39-79 g) were randomly allocated to treatments involving *ad libitum* feeding of 7 experimental diets. Diet 1 (basal) was a ground rat diet,[†] diets 4-6 were a ground rat diet containing .75, 1.5 and 3.0 μ g, respectively, of SC-6582[‡] per g of diet, and diets 7-9 were a ground rat diet containing .75, 1.5 and 3.0 μ g, respectively, of Ro-2-7239[‡] per gram of diet. Tap water was given *ad libitum*.

Statistical comparison of treatment means was done by using error mean squares, obtained from analyses of variance, to compute least differences required for significance (10).

Results. Data obtained by feeding normal and castrate male rats and normal female rats equal quantities of compounds SC-6582 or Ro-2-7239 are shown in Table I, Results of feeding, individually to appetite, normal male or female rats graded levels of the compounds SC-6582 and Ro-2-7239 are shown in Table II.

Our experiments show that both compounds tend to depress gain and feed efficiency. The depression of these responses reached significant levels in the case of compound Ro-2-7239 fed to normal male rats at the 1 μ g/g level employed in Exp. 1. No significant changes in size of pituitary or thyroid glands were observed upon feeding of either compound. Compound SC-6582 at the 3 μ g/g level significantly increased the size of adrenals of normal male rats. No significant changes in size of testes or of uterus were noted in the case of either compound.

Discussion. Administration of compound SC-6582 did not produce an increase in uterine weight/100 g of body weight, a finding at variance with the cited work(9). A considerably smaller total amount of substance administered in the present work may explain the lack of agreement.

Administration of compound Ro-2-7239 tended to reduce, or leave unchanged, the size of adrenals of female rats. This finding agrees with that of the previous work(8), but the tendency to increased size of these glands

in normal and castrate male rats is interesting. A tendency toward increased size of testes was also found with this compound and agrees with the results of the previously cited work.

Summary. Studies have been made of the effects of feeding 2 compounds, Ro-2-7239 (2-acetyl - 7 - oxo - 1,2,4,4a,4b,5,6,7,9,10,10a - dodecahydrophenanthrene) or SC - 6582 (17a - methyl - 17 - hydroxy - 5(10) - estren-3-one), to rats at levels of .75, 1.0, 1.5, or 3.0 μ g/g of diet. Equalized or *ad libitum* feeding was employed with weanling or young adult normal or castrate males or normal females. Compound Ro-2-7239, but not compound SC-6582, significantly inhibited both weight gain and feed efficiency in normal male rats. No significant changes in response to either compound were noted in castrate males or normal females. Compound SC-6582 at the 3 μ g/g level, but not compound Ro-2-7239, produced significant increases in size of adrenals in normal male rats. Neither compound at the 3 μ g/g level produced significant alteration in size of pituitary, thyroid, testes, or of uterus.

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Influence of Fatty Acids and Sterols on Atherosclerosis in the Avian Abdominal Aorta.* (26238)

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We have previously demonstrated that dietary corn oil retards the avian aortic atherosclerosis which develops both in presence and absence of dietary cholesterol(1,2). The present study was undertaken to (a) ascertain whether the fatty acids or the sterols in corn oil provided the observed protection and to (b) elucidate the importance of circulating and aortic cholesterol levels in relation to atherogenesis.

Experimental. Day-old Leghorn cockerels were fed a standard ration for 17 days, then were divided into 4 groups of 20 and given the experimental diets for 6 months. The basal ration contained in percent: soybean oil meal (50% protein) 50.0; alfalfa meal 2.5; dicalcium phosphate 2.5; corn oil 2.0; limestone with trace minerals (Mico concentrate, Limestone Corp. of America, Newton, N. J.) 1.0; salt 0.5; choline Cl 0.2; DL-methionine 0.2; B vitamin mix 0.1; A, D and E mix 0.1 (for composition of both mixtures see Fisher and Johnson(3)); antioxidant (Santoquin, Monsanto Chemical Co., St. Louis, Mo.) 0.02; and glucose to 100. The experimental design included the following: 1) control group receiving the unsupplemented ration, 2) a group receiving 2% cholesterol and an additional 5% corn oil, 3) a group getting 2% cholesterol and 0.05% corn oil sterol (approximately equivalent to sterol content of 5% corn oil) and 4) a group receiving 2% cholesterol plus 3% fatty acids (containing 72.9% linoleic acid which is approximately equivalent to the amount of linoleic acid in 5% corn oil). All supplements replaced equal amounts, by weight, of glucose. The 2% corn oil was included in the basal ration to facilitate absorption of the dietary cholesterol in the group of birds fed the sterol fraction of corn oil.

Plasma cholesterol was determined intermittently during the study. At the end of the 6-month experimental period all birds were killed, their aortas removed, visually graded, and the abdominal section of each aorta analyzed for total fat, cholesterol and polyunsaturated fatty acids by methods previously described(1). The aortic scoring system was designed primarily to evaluate degree of lumen encroachment by proliferative lesions, and secondarily to evaluate gross fatty infiltration, hardness and roughness of the intimal surface. No detailed analyses were carried out on the thoracic segments of the aorta since these showed negligible involvement for all groups.

Twice during the study, 24 hour collections were made of the excreta. These were dried, twice extracted for 24 hours on a shaker with chloroform-methanol(2:1), and cholesterol (sterol) determined on the filtered extracts by the method of Zlatkis *et al.*(4).

Results and discussion. The pertinent observations are given in Table I. Although the differences among groups are comparatively small, it appears that the group receiving the fatty acids equivalent to 5% corn oil had the greatest atherosclerotic involvement as judged by frequency of high scores, average score and average abdominal aortic weight. Conversely, the group receiving the sterols equivalent to 5% corn oil appears to have the least severe involvement. In this connection Beveridge *et al.*(5) working with the hypocholesterolemic effect of corn oil in humans, found at least part of the active principle to be present in the sterol fraction. In a similar vein, Howard and Gresham(6) have interpreted their results to mean that essential unsaturated fatty acids may facilitate deposition of cholesterol in tissues and production of atherosclerosis.

The group which received the fatty acid fraction also had the highest amount of aortic and plasma cholesterol, but it is note-

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TABLE I. Effect of Corn Oil and Fractions on Cholesterol-Induced Avian Atherosclerosis.

Measurements	Dietary groups			
	Control	2% cholesterol plus		
		5% corn oil	Corn oil sterol	Fatty acids
		Abdominal aorta		
Score	1.9 $\pm$.18*	1.9 $\pm$.09	1.8 $\pm$.06	2.0 $\pm$.08
Frequency of high scores†	6/18	6/18	5/19	10/20
Wt (mg)	64.9 $\pm$ 3.2	65.6 $\pm$ 3.7	67.7 $\pm$ 2.6	73.0 $\pm$ 2.7
Cholesterol (% of dry defatted tissue)	1.39 $\pm$.07	2.19 $\pm$.26	2.04 $\pm$.21	2.52 $\pm$.22
Fat (mg/g dry tissue)	130.1	85.4	89.4	113.3
Dienoic acid (% of fat)	16.7	22.2	15.1	23.9
Monoenoic acid (% of fat)	16.1	31.9	25.9	6.6
Saturated acid (% of fat)	60.9	37.9	51.6	63.9
		Plasma		
Cholesterol (mg %)	203 $\pm$ 16	464 $\pm$ 41	381 $\pm$ 40	478 $\pm$ 45
		Excreta		
Fat (% of dry matter)	4.3	12.4	10.2	11.6
Cholesterol (mg/g dry defatted excreta)	5.8	61.0	63.4	60.9
Cholesterol (% of fat)	13.5	49.4	62.1	52.3

* Mean $\pm$ stand. error.

† For this comparison, scores over 2.0, avg for the group showing greatest involvement, were used.

worthy that neither the aortic or plasma cholesterol levels among the other 3 groups bore consistent relationship to severity of atherosclerosis.

The polyunsaturated fatty acid analyses of abdominal aortic fat show the highest dienoic acid content for the groups that had received high dietary linoleic acid in the 5% corn oil—or the 3% fatty acid supplement. The high cholesterol content of aortic fat in the group receiving the fatty acids thus is not related to any essential fatty acid deficiency (7) since it is associated with the highest level of dienoic acid.

While dienoic acid content of the aortic fat reflects dietary intake, the monoenoic and saturated fatty acids for the corn oil *vs.* the fatty acid supplemented group show considerable variation. In the corn oil-fed birds the increase in dienoic acid clearly occurred at the expense of the saturated fatty acids, whereas in the fatty acid-supplemented animals the increase in dienoic acid was at the expense of the monoenoic acid only.

Attention is drawn to the much reduced deposition of total fat in the abdominal aorta in birds receiving corn oil sterol (as such, or with the whole corn oil supplement) as compared to control birds or those receiving the

fatty acid supplement. The significance of this observation in relationship to atherogenesis must await further experimentation.

The sterol excretion data in Table I demonstrate that dietary cholesterol was equally well absorbed in the 3 cholesterol-supplemented groups. Thus, lower blood and aortic cholesterol levels as well as lower severity of atherosclerosis in the animals fed the sterol-supplemented diet cannot be explained on the basis of interference with cholesterol absorption.

Summary and conclusion. Male chickens were fed for 6 months on rations containing (a) no supplement, (b) cholesterol plus corn oil, (c) cholesterol plus corn oil-sterol and (d) cholesterol plus fatty acids rich in linoleic acid. Atherosclerotic severity tended to be greatest in the group given fatty acids and least on the sterol-containing diet. Since sterol excretion indicated a similar rate of cholesterol absorption on the cholesterol-supplemented diets, it appears that the principle in corn oil which retards avian aortic atherogenesis is present in the sterol fraction.

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for providing corn oil sterols used in this study.

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Effect of Insulin on Serum Proteins in Scurvy.* (26239)

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It has been reported(1) that in scorbutic guinea pigs α_2 and β_2 globulins are diminished; α_1 -globulin is increased and no change takes place in β_1 and γ -globulins. Insulin is involved in biosynthesis of proteins (2) and hypoinsulinism has been observed in scurvy (3,4). The derangement of serum proteins in scorbutic guinea pigs might have been due to diminished insulin formation. It was, therefore, of interest to study the effect of prolonged injection of small doses of insulin on the protein pattern of serum in guinea pigs during the progress of scurvy. The carbohydrate and lipid containing proteins of normal, scorbutic and insulin-treated scorbutic guinea pigs were also determined.

Methods. Forty-eight male guinea pigs each weighing between 250 and 300 g were divided into groups of 3 animals, one normal, one scorbutic and one insulin-treated scorbutic. All the animals were fed a scorbutogenic diet(5). The amount of diet consumed per day by the scorbutic member of a group was fed to the other 2 members of the group on the subsequent day. The 'normal' animals were fed 5 mg ascorbic acid per animal per day. All the animals received 2 drops of a concentrate of Vit. A and D twice a week. From the 10th day on each animal of the insulin treated group was given a daily subcutaneous injection of protamine zinc insulin

(Lilly) in a dose increasing from 0.1 unit per 100 g body weight to 0.3 unit over the period of experiment. On the 24th day of experiment all the animals were sacrificed after an over-night fast, blood collected from neck veins, and clear serum analyzed for different protein fractions(6), glyco- and mucoproteins (7) and lipoproteins(8,9). Swahn(10) had shown that uptake of Sudan Black B by whole serum on filter paper was correlated with total lipid content of serum. Total area of the plotted curve was, therefore, equivalent to total serum lipid content of sample used for electrophoresis. The α and β -lipoproteins and the "o" fraction were expressed, on the basis of their respective areas, as percentages of the total area and hence of total lipid.

Results. Serum albumin and α_2 and β_2 globulins which diminished and α_1 globulin which increased in scurvy were brought back to approximately normal level after prolonged insulin treatment of the scorbutic animal. Serum glycoprotein and mucoprotein fractions which increased in scurvy remained unaffected after insulin treatment. Serum α -lipoprotein and "o" fractions considerably diminished and β -lipoprotein fraction significantly increased in the scorbutic condition. Insulin treatment did not produce marked effect on these fractions. The results are given in Table I.

Discussion. The alterations in serum protein patterns of scorbutic guinea pigs could

* This investigation was carried out with financial assistance of the Indian Council of Med. Research, New Delhi.

TABLE I. Different Fractions of Serum Proteins in Guinea Pigs.*

		Normal	Scorbutic	Insulin-treated scorbutic
Albumin		49.07 ± .48	42.38 ± .57	44.70 ± .86
Globulins— α_1	(% of total protein)	8.15 ± .11	14.78 ± .23	8.14 ± 1.69
α_2		26.10 ± .38	21.58 ± .32	25.68 ± .18
β_1		4.25 ± .05	4.26 ± .07	4.33 ± .07
β_2		6.85 ± .09	5.37 ± .22	6.85 ± .06
γ		5.68 ± .52	11.67 ± .69	10.35 ± 1.17
Total serum proteins (%)		5.43 ± .16	3.45 ± .23	4.17 ± .13
Glycoproteins (mg hexose/100 ml)		156 ± 4.03	194 ± 8.66	188 ± 5.02
Mucoproteins (<i>idem</i>)		24 ± 1.17	45 ± 1.67	55 ± 2.42
Lipoproteins (%)— α -fraction		36.8 ± .85	17.65 ± .33	18.17 ± .55
β " "		26.5 ± .85	65.70 ± .96	54.24 ± 2.89
"o" " "		36.6 ± 1.01	17.09 ± .95	28.63 ± .58

* Sixteen animals in each group. Values are mean ± S.E.

be brought back to almost normal levels after insulin treatment of the scorbutic guinea pigs for 14 days. The changes in the protein pattern in scurvy, therefore, might be due to insulin insufficiency associated with the condition. The increase in serum glyco- and mucoproteins of scorbutic guinea pigs might be due to an increased release of mucopolysaccharides from ground substances. In scurvy, serum α -lipoprotein is diminished whereas total of serum α_1 - and α_2 -globulins is not altered. The significant increase in β -lipoproteins with no change in total β -globulins in scurvy is possibly due to increased body serum cholesterol observed in this condition(11).

Summary. Serum albumin, α_2 - and β_2 -globulins which diminished and α_1 -globulin which increased in scurvy became approximately normal after insulin treatment. The changes in protein pattern of serum might be due to insulin deficiency of scorbutic guinea pigs. Serum glycoproteins, mucoproteins and β -lipoproteins increased and α -lipoprotein and "o" fraction diminished in scurvy.

Insulin treatment did not produce marked change in these fractions. Vit. C seems to have a specific role in metabolism of mucopolysaccharides.

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Influence of Hypophysectomy on Oxidative Enzymes and Size of Parietal Cells in Gastric Mucosa.* (26240)

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Although hypophysectomy of the rat results in a profound involution of the gastric chief cells and a fall in their capacity to secrete pepsinogen(1), the changes which occur in parietal cells is less clear. Crafts and Walker(2) reported a moderate elevation in pH of the gastric juice and reduction in total acidity; similar changes were observed in the dog(3). Structurally, Friedman(4) described a 50% reduction in number of parietal cells without presenting supporting evidence and Baker and Abrams(1) observed an irregular reduction in cell size with some loss of fuchsinophilic granules and mitochondria.

The objectives of this study were to observe after hypophysectomy (a) if a reduction in size of parietal cells is demonstrable by quantitative means, and (b) if alteration occurs in oxidative enzymes in the mucosal cells. The latter point is important because of the irregular mitochondrial depletion previously observed(1) in parietal cells and the depressed functional state of the chief cells.

Procedure. Young adult female Sprague-Dawley rats were fed Purina Laboratory Chow and starved 18 hours prior to being killed. At termination of the study, completeness of hypophysectomy was proven by microscopic examination of sections of the pituitary area.

For the study of parietal cell area, the rats were divided into 3 groups: (a) hypophysectomized, vehicle-treated; (b) hypophysectomized and treated twice daily with 125 μ g

somatotropin,‡ 15 μ g of corticosterone and 2 μ g of l-thyroxine (STC), and (c) nonhypophysectomized, vehicle-treated. The somatotropin was dissolved in distilled water at a concentration of 2 mg/ml, the l-thyroxine in water (40 μ g/ml), the hormone first being dissolved in one drop of 0.1 N NaOH, and the corticosterone was suspended in 0.9% NaCl (150 μ g/ml) with Tween 80 as the suspending agent. The 3 hormones were drawn into the syringe and injected together at one site. The nonhormonal ingredients of the injection media were administered to groups a and c in similar manner and volume. Fifteen or 16 days elapsed between hypophysectomy and initiation of hormone treatment; treatment was continued for 15-16 days. Groups b and c received daily the amount of food consumed by Group a on the previous day. When killed, the stomach was expanded before removal by injection of 2.5 ml of formol-alcohol-acetic acid fixative. After fixation for 48 hours, strips of glandular mucosa which extended along the greater curvature from the horizontal ridge to the pylorus were embedded, sectioned, and stained with periodic acid-leucofuchsin and methylene blue.

Outlines of parietal cells were drawn with a camera lucida at a magnification of 1900 $\times$. The areas studied were in the region of the neck and isthmus of the gastric glands, these areas being selected at regular intervals throughout the section. All parietal cells in each area which were cut through the nucleus were drawn. The area of each cell outline was determined with a planimeter. An average of 52 to 54 cells in each of the 3 groups was measured.

Oxidative enzymes were studied in the mucosa of 8 pairs of hypophysectomized and nonhypophysectomized rats, the latter being pair-fed against the operated animal. Mean initial body weight for the nonhypophysecto-

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TABLE I. Effect of Hypophysectomy and Replacement Therapy on Area of Parietal Cells.

Group	No. of rats	Mean body wt (g)		Mean area of parietal cells (μ^2)	Probability of error*
		Initial	Final		
1. Hypophysectomized, vehicle	9	154 $\pm$ 2†	148 $\pm$ 3	122 $\pm$ 5	1 vs 2 <.02
2. Hypophysectomized, STC‡	9	158 $\pm$ 2	172 $\pm$ 5	141 $\pm$ 5	2 " 3 <.05
3. Nonhypophysectomized, vehicle	10	159 $\pm$ 1	182 $\pm$ 3	163 $\pm$ 8	1 " 3 <.001

* Student-Fisher t.
corticosterone.

† Stand. error of mean.

‡ STC = somatotropin, thyroxine and

mized rats was 173 $\pm$ standard error 2 g and for the hypophysectomized rats 172 $\pm$ 4 g; final weights were 202 $\pm$ 2 and 148 $\pm$ 3 g, respectively. Twenty-nine to 80 days elapsed after the operation before killing. Pieces of mucosa were frozen over liquid nitrogen and sectioned at 6 μ in a cryostat. The following histochemical reactions were carried out on these sections: succinic dehydrogenase (SD)(5), lactic, malic, isocitric and α -glycerophosphate dehydrogenases (LD, MD, ID, GD)(6), diphosphopyridine nucleotide diaphorase (DPND)(7), and cytochrome c oxidase(CO)(8).

Observations and discussion. Hypophysectomy reduced the mean area of parietal cells from a mean of 163 $\pm$ 8 μ^2 to 122 $\pm$ 5 μ^2 , this difference being significant at the 0.1% level (Table I). Treatment of hypophysectomized rats with STC caused a significant increase in area but did not restore it to the level seen in nonhypophysectomized controls.

In nonhypophysectomized rats the parietal cells stained intensely with all of the enzyme procedures (Figs. 1A and C) except that for ID. With ID the cytoplasm was a faint pink and sparse, formazan granules appeared in at least the larger parietal cells. GD was weaker than the other enzymes. The stain for CO appeared as densely packed fine granules; the other procedures which utilized nitro blue tetrazolium revealed large globular and finer formazan bodies, at least the latter appearing to be mitochondria.

Chief cells of control rats stained much less intensely (Fig. 1A and D), for the most part only fine mitochondria being revealed. Again only ID failed to be revealed clearly.

The mucous epithelial cells of the mucosal surface and foveolae stained strongly with all procedures except that for ID. These cells were unique in showing a prominent concentration of activity in the cytoplasm immediately above the nucleus.

Finally the technics for dehydrogenases and DPND revealed large globular bodies in the superficial epithelium and underlying connective tissue located between foveolae. The significance of these bodies is not understood.

Hypophysectomy did not selectively suppress any of the enzymes studied in any of the 3 cell types observed. The overall quantity of activity for all enzymes was reduced somewhat in the parietal cells in association with reduction in cell size. However, parietal cells continued to stain intensely as illustrated by the preparations for CO (Fig. 1D and DPND (Fig. 1B). Since chief cells involute markedly after hypophysectomy, their total activity for each enzyme was reduced (Fig. 1B and D). However, they still contained all of the enzyme activities and in some cases the staining intensity was even greater than that in the controls.

These observations suggest the presence of a general depression in oxidative activity of parietal and chief cells in the hypophysectomized rat which could be associated with the reduced production of HCl and pepsinogen. However, none of the enzymes studied was affected to a greater degree than the others.

Summary. Gastric parietal cells were reduced in size after hypophysectomy of the rat. Treatment with STC enlarged the cells but did not restore their normal size. Histochemical demonstration of cytochrome c oxi-

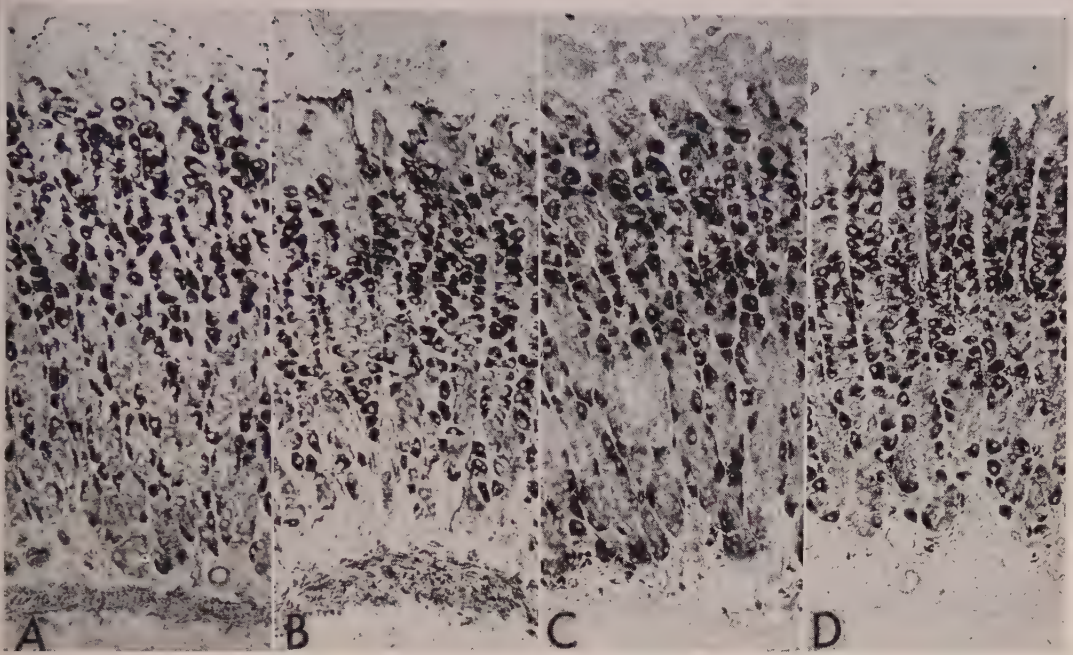


FIG. 1. In all cases the black cells are parietal cells and the gray ones deep in the glands are chief cells. A and B, DPN diaphorase; C and D, cytochrome c oxidase. A and C were prepared from a nonhypophysectomized control; B and D from a rat which had been hypophysectomized 29 days. In B and D the parietal and chief cells are smaller and their total activity somewhat less, these changes being more marked in the chief cells.

dase, DPN diaphorase and 5 dehydrogenases did not reveal any one which was affected more than the others in either parietal or chief cells. In both cell types some overall reduction in activity occurred coincident with a reduction in cell size.

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Studies of Alcaptonuria: Binding of Homogentisic Acid Solutions to Hide Powder Collagen.* (26241)

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Data have been presented previously to indicate that unoxidized homogentisic acid solutions can interact with hide powder collagen preparations(1). It has also been shown (2) that the interaction with unoxidized homogentisic acid (HGA) is largely a reversible phenomenon, whereas interaction with polymerized, autoxidized homogentisic acid solutions (HGA-OP) presumably involves actual binding to collagen. The present studies were undertaken in an attempt to provide quantitative data on these interactions.

Materials and methods. One hundred milligram samples of commercially available limed hide powder collagen (American Standard Hide Powder[†]), neutralized to the isoelectric point (pH 5.4) were passed through No. 20 ASTM sieves and placed in chemically clean, dry cellulose ultracentrifuge tubes.[‡] Ten ml of each of the test solutions (*vide infra*) were then layered on to the collagen preparations and thoroughly stirred to effect dispersion of the solid phase. The tubes were permitted to stand in air at room temperature (25°C) for 24 hours and from 1 to 2 weeks, with occasional stirring. At stipulated intervals (Table I), tubes were centrifuged in a Spinco analytical ultracentrifuge at 30,000 rpm ($59,310 \times g$) for 5 minutes. The particle-free supernatants were decanted and their absorbance determined using a Beckman DU spectrophotometer with photomultiplier attachment.

Similar studies were performed using acetylated hide powder collagen and Lyon poudre de peau blanche (Inst. de Recherches

pour les Industries du Cuir, Lyon, France).[§] The acetylated collagen was prepared by reacting isoelectric American Standard Hide powder collagen with acetic anhydride in fully saturated sodium acetate as described previously(1,3,4).

The test solutions were D₂O,[¶] freshly prepared 0.03 M HGA, and HGA-OP prepared by autoxidation of 0.03 M HGA solutions. All solutions were employed in a concentration range such that physical or optical density of each adhered to the Beer-Lambert law.

Physical density of D₂O solutions was determined by the falling-drop method(5) using xylene: bromobenzene mixtures (79:21) for the liquid phase and a specially designed, calibrated microliter syringe assembly which gave reproducible readings to the fifth decimal place. Optical density of HGA solutions was determined from absorbance observations at the absorption maximum at 2900 Å or along the linear slope of the absorption peak at 3250 Å. HGA-OP solutions were read at 3750 Å.

Binding was determined by the percentage change in density of the supernatant liquid phase before and after the period of interaction with each adsorbent by:

$$\% \text{ "bound" } = \% \text{ bound} / 100 \text{ mg adsorbent} \\ = \frac{100 (D_o - D_f)}{D_o}$$

where, D_o = initial density (optical or physical) of liquid phase, and D_f = final density, after interaction with the adsorbent.

Initial (pH_i) and final (pH_f) pH values of the supernatant liquid phase were determined using a Beckman zeromatic pH meter

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[†] Purchased from Frank F. Marshall, Ridgeway, Pa.

[‡] No. 2235, $\frac{5}{8} \times 3$ ins., purchased from Spinco Beckman, Palo Alto, Calif.

[§] Kindly supplied by Prof. K. H. Gustavson, Swedish Tanning Research Inst., Stockholm.

[¶] Purchased from Volk Radiochemical Co., Chicago, Ill.

TABLE I. Extent of Binding of Unoxidized, Monomeric Homogentisic Acid (HGA) and Autoxidized, Polymeric Homogentisic Acid (HGA-OP) Solutions to American Standard Hide Powder Collagen (HPC). Acetylated hide powder collagen indicated by AcHPC and deuterium oxide by D₂O.

Reactants	Time	pH _i	pH _f	Δ pH	% bound
HGA + HPC	24 hr	3.32	3.35	+ .21	3.0
D ₂ O + HPC	"	3.52	4.65	+1.13	.01
HGA-OP + HPC	"	3.37	3.57	+ .20	69.3
HGA-OP + AcHPC	"	3.36	3.47	+ .11	66.2
HGA-OP + HPC	1 wk	3.33	3.85	+ .52	68.0
HGA-OP + HPC	2 "	3.35	4.62	+1.27	62.2
HGA-OP + AcHPC	2 "	3.35	3.85	+ .50	62.1

pH_i of all solutions was not adjusted except for that of D₂O. This was adjusted from pH 5.4 to pH 3.5 by addition of HCl (0.1N).

(instrumental error 0.03 pH).

Results. The extent of binding of each of the test solutions to American Standard hide powder collagen preparations at pH approximately 3 is given in Table I. Results with Lyon poudre de peau blanche were essentially identical and are not included here.

Under the described experimental conditions, it is quite clear that: (1) neither D₂O nor HGA bind to hide powder collagen to any appreciable extent; (2) there is significant binding of HGA-OP solutions to hide powder collagen; (3) HGA-OP binding is unaffected by acetylation of the collagen amino acids, and (4) HGA-OP binding is essentially unaffected by the time of interaction with the hide powder collagen preparations.

Discussion. These observations on the extent of binding of the various homogentisic acid solutions by hide powder collagen corroborate previous qualitative data on collagen swelling effects of homogentisic acid solutions(1) and semi-quantitative observations on adsorption of homogentisic acid solutions on to collagen chromatographic columns(2).

Furthermore, these data provide confirmation of the earlier inference that although monomeric, unoxidized homogentisic acid interacts with hide powder collagen in the pH range below that of the isoelectric point of the collagen preparation, this particular interaction is markedly pH-dependent and does not in fact involve a binding mechanism. Since it has been shown previously that there is no appreciable interaction between these 2 moieties at pH's at or above the isoelectric

point, it would appear unlikely that monomeric homogentisic acid would have much if any effect on collagen *in vivo*.

With autoxidation of the colorless monomer to the pigmented oxidation polymer(s), however, there is a distinct and unequivocal difference in the observed interactions with collagen. Pronounced binding of polymerized homogentisic acid solutions (HGA-OP) to the collagen occurs quite irrespective of the pH of the HGA-OP solutions. In these instances, observed swelling effects are more or less irreversible, even after removal of the HGA-OP solution, are entirely independent of pH and are associated with little or no solubilization of collagen(1). Indeed, HGA-OP has all characteristics, including chemical binding as noted here, of a potent tanning agent for hide powder collagen. This tanning property, which is entirely lacking in the unoxidized monomeric solutions (HGA) is present not only at pH values, but also at ionic strengths which are characteristic of body fluids.

It is not unreasonable to suggest, therefore, that this tanning phenomenon may account for the morphological sequellae observed in alcaptonuria. In such a view, ochronosis and arthritis in patients with homogentisic acid retention (alcaptonuria) could arise in consequence of some mechanism (other than tyrosinase catalysis(6)) which brings about polymerization of HGA to HGA-OP, which, when once formed, then could chemically bind to collagen and result in collagen tannage.

Since acetylation of the collagen amino acid groups did not appreciably alter the ex-

tent of HGA-OP binding, the chemical bond formed between the two moieties presumably would be primarily of the non-ionic, coordinate (hydrogen bond and van der Waal's) type. For steric reasons, the tanning agent (HGA-OP) would probably act as a multi-functional agent to form such primarily coordinate inter-chain cross-links between adjacent collagen polypeptide chains(2). The net effect would be stabilization and "hardening" of the collagen lattice structure.

Summary. Binding of monomeric, unoxidized (HGA) and polymeric, autoxidized (HGA-OP) homogentisic acid solutions to hide powder collagen preparations has been studied. It has been shown that there is no active binding of HGA to collagen, whereas there is extensive binding of HGA-OP to collagen. Coupled with previously presented

observations, it is suggested that HGA-OP can act as a potent tanning agent for collagen. It is further suggested that ochronosis and degenerative joint disease observed in patients with alcaptonuria may possibly be explained by the *in vivo* occurrence of such a phenomenon.

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Anaphylaxis in *Bordetella pertussis*-Treated Mice. IV. Schultz-Dale Reaction.* (26242)

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Injection of *Bordetella pertussis* cells into mice increases the susceptibility of these animals to fatal anaphylactic shock, whether the shock is induced by passive or active means(1). *B. pertussis* cells also increase susceptibility of mice to various agents and stresses such as histamine, serotonin, cold stress, x-rays, etc.(1). *B. pertussis* does not, however, increase susceptibility of the mouse skin to passive cutaneous anaphylaxis (PCA) (2). The mechanism by which *B. pertussis* produces the changes observed is not well understood, although *B. pertussis* may indirectly interfere with adrenal function(3). *B. pertussis* enhances antibody response to various antigens(4,5), and most likely increases the susceptibility of mice to active anaphy-

laxis by stimulating antibody response to sensitizing antigen. In spite of the adjuvant activity of *B. pertussis*, the relationship of this effect to the increased susceptibility to anaphylaxis produced in mice by these organisms has been questioned(4,5). Irrespective of the role that the adjuvant effect has on active sensitization, the increased susceptibility to passively induced anaphylaxis cannot be explained by the adjuvant effect of *B. pertussis*, since in this case it can be shown that *B. pertussis*-treated mice become highly sensitive to fatal anaphylaxis with amounts of antibody that do not sensitize normal mice to fatal shock(3,6). In this paper the effect of *B. pertussis* cells on specific active and passive sensitization of smooth muscle will be described.

Materials and methods. Mice. Female mice of the CFW strain weighing from 14 to 18 g purchased from Carworth Farms, New

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City, N. Y., were sensitized with crystalline egg albumin (Ea), and the uterus subsequently used for the Schultz-Dale test. *Antigen*. The Ea used was 5 times recrystallized egg albumin prepared in our laboratories by the method of Kekwick and Cannan as outlined by Kabat and Mayer(7). *Antibody*. Two pools of mouse peritoneal fluid were used, one containing 151 μg and the other 2001 μg of antibody nitrogen (Ab N) per ml. These fluids were prepared by a method previously described(8). Antibody determinations were made by the method of Gitlin(9). A pool of rabbit anti-Ea serum containing 481 μg of Ab N/ml was also employed in some experiments. *B. pertussis cells*. Smooth *B. pertussis* cells grown in the liquid medium similar to that of Verwey *et al.*(10) and killed by addition of 0.01% thimerosal (merthiolate) were employed. *Schultz-Dale reaction*. The Schultz-Dale reaction was carried out in the tissue bath at 37°C. The Tyrode's solution employed in the bath was of the following composition:

Sodium chloride	8 g
Potassium "	.2 g
Calcium "	.05 g
Magnesium "	.1 g
Sodium dihydrogen phosphate	.05 g
Sodium bicarbonate	1 g
Glucose	1 g
Distilled water	1000 cc

pH of this solution was 7.6

Usually after mixing the reagents in the order stated, the solution was kept at least overnight at room temperature before using. In freshly made Tyrode's solution, the uterine muscles did not, in many cases, relax sufficiently to avoid nonspecific, spontaneous contractions. The uterine horn, connected to a kymograph by means of a lever which pulled the muscle strip gently, was allowed to remain in the bath until no spontaneous contractions occurred. Repeated washings of over-active muscles with Tyrode's solution usually stopped nonspecific contractions. Once the muscle had completely relaxed, the antigen (Ea) was added in a concentration of 0.05 mg per ml of bath. The mice employed were killed by breaking the spinal cord at the neck region and uteri were re-

TABLE I. *In Vitro* Anaphylaxis with Uteri from Passively Sensitized Mice.

Source of antibody	μg anti-Ea Ab N/mouse	Results*
Rabbit	96	7/10†
"	9.6	0/10
Mouse	2	10/10

* All muscle strips that had negative Schultz-Dale reactions contracted with 10 μg of acetylcholine/ml of bath.

† Numerator = No. of muscles with positive Schultz-Dale. Denominator = Total No. of muscles tested.

moved and placed in Tyrode's solution. These muscles could be stored for at least 24 hr at 2-4°C without affecting their reactivity. Muscles that did not give a specific contraction when antigen was added were subsequently challenged with 0.05 μg of serotonin or 10 μg of acetylcholine per ml of bath to test their reactivity.

Experimental results. Exp. 1. Passive sensitization with mouse and rabbit antibody. Each of 10 mice was passively sensitized with 2 μg of anti-Ea Ab N injected intraperitoneally and 6 hr later the uterine horns were removed and tested in the tissue bath for their ability to exhibit the Schultz-Dale reaction. In addition, 10 mice received 96 μg of rabbit anti-Ea Ab and 10 received 9.6 μg . The results presented in Table I show that all uterine strips of mice which received 2 μg of mouse Ab N gave a positive contraction while none of the strips from mice which received 9.6 μg of rabbit Ab N showed any sensitivity. Of those mice receiving 96 μg Ab N, only 7 out of 10 strips tested gave definite positive reactions; the remaining 3 were doubtful.

After this preliminary experiment the following experiment was performed to determine exactly how much mouse Ab N was required to produce the Schultz-Dale reaction and to show whether pre-treatment with *B. pertussis* cells affected this sensitivity.

Exp. 2. Quantity of mouse antibody required for Schultz-Dale reaction in normal and B. pertussis-treated mice. To groups of 5 mice each, various amounts of antibody were given 24 hr before removal of the uterus. For each antibody level, 2 groups of mice were used: one normal group and the

TABLE II. Sensitivity of Uteri from Normal and *B. pertussis*-Treated Mice Receiving Various Amounts of Mouse Antibody.

μg of anti-Ea Ab N/mouse	Results	
	Uteri from normal mice	Uteri from <i>B. pertussis</i> - treated mice
.0625	0/5*	0/5
.125	3/5	2/5
.5	4/5	4/5
1.0	4/5	4/5
2.0	5/5	5/5
4.0	5/5	5/5
8.0	5/5	5/5

* See Table I.

other treated 4 days before injection of the antibody with approximately 2 billion *B. pertussis* cells given intraperitoneally (Table II). Five of 10 uteri from mice receiving as little as 0.125 μg of mouse Ab N gave a Schultz-Dale reaction and 8 uteri of 10 mice receiving 0.5 μg Ab N gave positive Schultz-Dale reactions. Larger amounts of Ab produced sensitization of nearly all the uteri. No difference was found in quantity of Ab required to sensitize the uteri of either *B. pertussis*-treated mice or normal mice. All the uterine strips which failed to exhibit the Schultz-Dale reaction were tested with 0.05 μg of serotonin per ml of bath and found to be active.

Exp. 3. Development of Schultz-Dale reaction in normal and B. pertussis-treated mice after active sensitization with 0.5 mg of egg albumin. Forty mice received 2 billion *B. pertussis* cells mixed with 0.5 mg of Ea. Groups of 10 *B. pertussis* and Ea-treated mice and 5 of those that had received Ea alone were killed at different intervals of time after sensitization. Uterine horns were removed and challenged in the tissue bath as before (Table III). Uteri from mice in Groups 1, 2, 3 and 4 were tested 5, 7, 8 and 10 days, respectively, after administration of the sensitizing dose. As controls the uterine horns of 4 normal unsensitized mice were included with each of the 4 groups. Uterine horns obtained 5 days after injection of the antigen alone or with *B. pertussis* did not contract. On the seventh day, however, 3 of 10 uterine horns were found to be sensitive in the *B. pertussis*-treated group while none

in the group which received Ea alone responded to Ea. Eight days after the sensitizing dose, 9 of the 10 uterine strips of the *B. pertussis* groups were found to give the Schultz-Dale reaction while only 1 of the 5 Ea sensitized mice responded to Ea. On the tenth day, all uterine strips tested in either groups had developed sensitivity to Ea. None of the control strips from unsensitized mice contracted after addition of antigen.

Discussion. The results presented here again point out the importance of employing homologous antibody in study of anaphylactic reactions in mice and perhaps all animals. As has been previously noted, rabbit antibody is far less effective in sensitizing mice to both fatal and passive cutaneous anaphylaxis (2,6). The present results show that rabbit antibody is also inferior in sensitizing uterine muscle of mice than is mouse antibody.

It was of interest to find that *B. pertussis* cells do not affect the sensitivity of muscle strips of mice receiving the preformed antibody in passive sensitization experiments. These results, as well as those obtained with respect to PCA, indicate that *B. pertussis* cells do not affect directly the reactivity of tissues, but rather that they affect other physiological, pharmacological or metabolic functions of the mouse. In the case of active sensitization of mice, *B. pertussis* accelerated the appearance of sensitivity of the muscle. This observation can be explained by the known adjuvant action of *B. pertussis* cells.

Although the present results do not elucidate the mechanism by which *B. pertussis* increases the susceptibility of mice to passive anaphylaxis, they do indicate strongly that

TABLE III. Development of *In Vitro* Anaphylactic Sensitivity in Normal and *B. pertussis*-Treated Mice Actively Sensitized with Ea.

Time after sensitiza- tion, days	Results*		
	Normal sensitized	<i>B. pertussis</i> - treated	Normal unsensitized
5	0/5†	0/10	0/4
7	0/5	3/10	0/4
8	1/5	9/10	0/4
10	5/5	10/10	0/4

* All muscle strips that gave a negative Schultz-Dale reaction responded to 10 μg of acetylcholine per ml of bath.

† See Table I.

B. pertussis does not interfere with localization of antibodies in tissues and that it does not make the tissues hypersensitive to an antigen-antibody reaction. This has been previously suspected since it has been observed that, although there is a marked difference in amount of antibody needed to produce fatal anaphylaxis, there is hardly any observable difference in amount of antibody required to produce anaphylactic symptoms such as ruffling of the hair, forced respiration, slight cyanosis, lethargy, etc. (3).

Summary. Mouse antibody was found to be more effective than rabbit antibody in sensitizing the mouse uterus to the specific antigen. It was also found that *B. pertussis* cells do not affect the sensitization of the mouse uterus when the mouse is sensitized by passive means. When the active method of sensitization of mice was employed, however, *B. pertussis* accelerated the appearance of sensitivity of the uterus as demonstrated by

the Schultz-Dale reaction.

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New Phages Active Against Staphylococci that Are Resistant to the Conventional Phage Set.* (26243)

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During studies on staphylococcal infections at Boston City Hospital, it was found that a large and probably increasing number of infections were caused by staphylococci that were insensitive to the phages generally used for typing, even at concentrations of 1000 x the routine test dilution (RTD) (1). A large proportion of these strains had been cultured from serious infections such as septicemias and pneumonias and they were usually resistant to several of the antibiotics in general use. The isolation and some of the properties of 5 new phages that were

found to be useful in typing these "nontypable" (NT) strains is described in this paper. Some findings with 2 other new phages related to 80/81 are also included.

Isolation of New Phages. For this purpose 108 nontypable strains cultured from different sources and with a varying antibiotic sensitivity were selected. They were all cross-cultured by the method of Fisk (2). An overnight culture of each strain was flooded on plates of trypticase soy agar and the inoculum was allowed to dry. A drop of a broth culture of each of the other strains was then applied on the surface in a similar manner as in the bacteriophage typing. The plates were read after incubation at 30°C for 18 hours. Some (24 of 5040 combinations) of these cross-cultures showed evidence of lysis. The areas of good lysis (7 in all) were

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‡ With technical assistance of Mildred W. Barnes; Dr. Bosko Postic also assisted in collection of some of the strains.

scraped off together with some of the underlying agar, suspended in 2 ml broth and shaken vigorously. Each supernate, after centrifugation at 3000 rpm for 20 minutes was spotted on the 2 members of the combination, on a new plate, to determine the lysogenic (resistant) and the indicator (sensitive) strain. After filtration to remove the lysogenic strain, the phage was purified by picking a single plaque which was further propagated.

Propagation of Phages. After a few passages on plates to increase the yield of phage, propagation in broth was used and proved satisfactory. To 20 ml broth in flasks was added 1 ml sterile solution of 1% CaCl_2 , 0.2 ml 4-hour broth culture of the propagating strain and 0.5 ml of undiluted phage. The cultures were incubated at 37°C for 6 hours. At that time a moderate growth was generally observed. After storage at 4°C overnight the cultures often turned clear. Serial 10-fold dilutions of the supernates after centrifugation were then tested on the propagating strain. A satisfactory titer was thus obtained.

Each of the 7 phages thus isolated was tested on all the new propagating strains, all of which were resistant to the conventional phages; 5 of the new phages proved to be distinct, each lysing only its own propagating strain. These 5 phages were selected for further study; their origins were:

Provisional designation of phage	Lysogenic strain		Indicator strain	
	No.	Source	No.	Source
A	206/60	Sputum	38/60	Sputum
B	258/60	Abcess	57/60	Stool
C	187/60	Sputum	113/60	Sputum
D	286/59	Urine	157/59	"
E	199/60	"	222/60	Abcess

Two other phages, provisionally called F and G, were isolated from colonies of the original culture plates of clinical specimens; both had caused a motheaten appearance of a few among the many colonies of *Staph. aureus* on these plates.† Phage F was ob-

tained from a motheaten colony grown from an ear discharge by suspending it in a small amount of broth. After vigorous shaking, the suspension was centrifuged; colonies of normal appearance on the same plate proved to be sensitive to the phage in the supernate, in contrast to the subculture from the nibbled colony which was resistant. A normal looking colony was used as the propagating strain (PS) and the phage propagated as described above. With the standard phages, both the normal colony (the PS) and the nibbled colony (the carrier) were lysed by phage 80 and weakly by phage 52.

Phage G was isolated in the same manner from a sputum culture. A few colonies on a plate had a motheaten appearance while the rest looked normal; the latter were sensitive to the phage whereas the nibbled colony was not. A normal colony was chosen for further propagation of the phage; it typed with the conventional set of phages as 80/81/KS6/42B, but the carrier colony was lysed only by phage 80. PS-F was lysed by phage F but not by G; PS-G was lysed by F and G, both of which proved to be closely related to 80 and 81 and they nearly always gave identical lysis of a large number of strains tested. Phage G thus appears to be of little significance and was eventually discarded.

Results. Lytic spectrum of new phages, as compared to standard phages. The lysis produced at RTD by the new phages and the standard set of phages used at this laboratory (3-5) on the propagating strains of all these phages is shown in Table I. Besides full lysis of their own PS, the new phages showed the following spectrum: Phage A lysed PS-6 and 83; phages B and E lysed no others; phage C produced weak lysis in PS-6 and 7; phage D lysed PS-83; phage F lysed PS-80, 81, 73, 42B and G; and phage G lysed PS 80, 81, 73 and (weakly) 42B.

PS-A, B, C, D, and E were each lysed only by its corresponding phage; PS-F was lysed by phages 80 and F, and PS-G by 80, 81, KS6 and G. The phages A to E thus have a narrow host range which would make them suitable for typing purposes. A, C, and D, seem to be related to the group III phages, whereas F and G are related to phages 80

† We are indebted to Miss A. Kathleen Daly, Bacteriology Lab., Boston City Hospital, for detection of these phages.

TABLE I. Lytic Spectrum of 7 New Phages on Their Propagating Strains: Comparison with Conventional Phages.

Propagating strains	Conventional phages																							New phages											
	29	52	52A	79	80	81	3A	3B	3C	55	71	6	7	42E	47	53	54	73	75	77	83	42D	187	42B	KS6	A	B	C	D	E	F	G			
29	+																																		
52		+																																	
52A/79			+		—																														
80				+	+	+																													
81					+	+																													
3A							+																												
3B								+	—	+																									
3C								+	+	+																									
55									—	—																									
71								+	—	—																									
6												+																							
7													+																						
42E															+																				
47																—	+	+	+																
53																	—	—																	
54																	+	+	+																
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F																																			
G																																			

+ = good lysis; — = weak or poor lysis.

and 81 and phages B and E did not lyse any other PS.

Typing with new phages of strains nontypable with standard phages. 158 strains isolated in 1959-1960 and nontypable with the standard phages even at 1000 $\times$ RTD were tested with the new phages A through G. Since phages F and G gave almost identical results, the latter is not included in the following analysis. Most of these untypable strains were identified during another study (1); 123 were from clinical sources (22 from outpatients and 101 from hospitalized patients) and 35 were obtained at autopsy. The results are listed in Table II. Of the 158 strains, 70 were lysed by phage A alone; 35 of these by the RTD and 35 only by 1000 $\times$ RTD; 4 to 11 strains were lysed by each of the other new phages. Only 7 strains were lysed by more than one of these 6 phages; 42 (27%) of the conventionally nontypable strains were negative with the new phages as well. Type A was particularly common among the strains isolated from autopsy material, representing 72% of all nontypable autopsy strains; the negatives, on the other hand, predominated among strains from outpatients. The hospitalized cases of type A infections were distributed among 19 wards, 8 of them in a single medical ward, and strains from the latter were resistant to all the common antibiotics except novobiocin.

Antibiotic sensitivity of strains of types A

TABLE II. Susceptibility to 6 New Phages of Pathogenic Staphylococci Nontypable with Conventional Phages (1000 $\times$ RTD).

New phages lysed	Source of strains			Total	
	Clinical* OP	IP	Autopsy	No.	%
A†	1	44	25	70	44.3
B	2	9		11	7.0
C		7	2	9	5.7
D		5		5	3.2
E	1	3		4	2.5
F	2	7	1	10	6.3
Misc.‡	2	5		7	4.4
Neg.	14	21	7	42	26.6
Total	22	101	35	158	100
% neg.	64	21	20	27	

* OP = outpatient case; IP = inpatient (hospital case).

† Includes those lysed weakly (or only by 1000 $\times$ RTD).

‡ Various combinations of these phages

TABLE III. Antibiotic Resistance of Strains Nontypable with Conventional Phages but Lysed by 6 New Phages.

New phages lysed	No. of strains tested	No. resistant to:*					
		P	S	T	CM	E	N
A†	70	68	68	69	30	66	21
B	11	8	0	0	0	0	0
C	9	9	8	8	7	8	6
D	5	5	2	3	1	2	1
E	4	0	0	0	0	0	0
F	10	6	3	3	2	2	1
NT	42	21	6	7	2	6	0

* Not inhibited in a 2-fold dilution test by (μ g/ml): Penicillin 0.8 (P); streptomycin 12.5 (S); tetracycline 12.5 (T); chloramphenicol 25 (CM); erythromycin 1.6 (E); novobiocin 1.6 (N).

† Includes those lysed weakly or by 1000 $\times$ RTD.

to F was tested with 2-fold dilutions in agar, using an inoculum of about 10^6 organisms in a replica-inoculating apparatus(6). The results are summarized in Table III. Strains of each type generally had a characteristic antibiotic spectrum. Type A strains were nearly all resistant to penicillin, streptomycin, tetracycline and erythromycin; 43% of them were also resistant to chloramphenicol and 30% to novobiocin. Strains of type B were generally resistant to penicillin, but sensitive to the others; those of type C were generally resistant to all antibiotics, whereas most of those of type F and all of type E were sensitive. These results suggest the possible usefulness of the new phages for typing.

Activity of the new phages on strains typable with the standard phages. 1. *Relation to "Group 80/81."* 116 strains that previously had typed as 80/81/KS6, 80/KS6, 80, 80/81, 81 or KS6 (here referred to as "group 80/81" for convenience) were tested with the new phages. All except 4 strains were lysed by phage F; 108 were lysed only by F, and 4 by another of these phages in addition. Two strains were lysed only by phage B and 2 were not lysed by any of them. These results demonstrate the close relationship of phage F to phages 80/81; the identity is not complete, however. Ten strains that did not

|| For definition of "resistance" see footnote in Table III. Values chosen represent the point where the cumulative percent distribution curve of sensitivity has flattened out, as shown previously(3).

TABLE IV. Activity of 6 New Phages on 100 Strains of Conventional Phage Patterns Other Than "Group 80/81."

Pattern with conventional phages	No. of strains tested	Pattern with new phages†
<i>Group I</i>		
29*	8	B(1), B/F(3), F(1), NT(3)
52	2	B(2)
52A/79	11	B(10), D(1)
79	3	B(3)
<i>Group II</i>	10	NT(10)
<i>Group III</i>		
42E	6	B/D(2), NT(4)
6/47	2	NT(2)
7	11	C(9), C/D(2)
47/53/83	2	A(2)
53	8	A(8)
54	9	F(5), NT(4)
77	9	C(9)
6/47/53/54/75/77/83	4	C/D(4)
Others†	12	A/B/C/D(1), B(1), B/D(1), C(1), D(1), F(1), NT(6)
<i>Miscellaneous</i>		
187	3	NT(3)

* Also includes those lysed by other phages in addition.

† 10 different patterns of Group III phages.

‡ No. of strains in parentheses.

respond to any standard phages, including 80, 81 and KS6, were lysed by phage F (Table II).

2. *Relation to other phage groups.* One hundred randomly chosen strains of a variety of other phage types were similarly tested with phages A to F; a variety of responses emerged as shown in Table IV. Most strains in phage Group I were lysed by phage B. All 10 Group II strains tested were negative. Among 63 strains of Group III, 26 were lysed by phage C, 11 by A, 11 by D, 6 by F and 4 by phage B. Phage E which was active against only a small number of nontypable strains (Table II) had no activity against any strains of these 2 series.

Discussion. Phage typing is an important tool in study of staphylococci, particularly in epidemiological surveys. In the last few years the marked predominance of 80/81, often considered as a single type, has, however, somewhat decreased the value of this method. Another limitation is the nontypable strains; an appreciable proportion of

strains are nontypable with this method and thus do not permit conclusions regarding epidemiological significance. The frequency of the nontypable strains generally varies between 10 and 20%. Since nontypable strains were most often recovered from healthy nasal carriers and less frequently from clinical lesions, they have been considered less virulent, and not much attention has been given to them.

A study of staphylococci recovered from patients admitted to Boston City Hospital from Sept. 1959 to Feb. 1960 disclosed a large, and probably increasing number of strains resistant to the standard set of phages used (20-30% of all strains). The infections caused by these organisms were often severe and included septicemias, pneumonias, etc. and this was also reflected by the high frequency of nontypable strains in postmortem cultures made at autopsy. Moreover, these staphylococci often turned out to be resistant to most or all of the antibiotics in general use.

The need for an improved phage typing technic was thus considered to be great. Of the two simplest methods available to find new phages, namely, adaptation of a known phage to a strain with low sensitivity to it or isolation of new "wild" phages, the latter method was chosen. For the crosscultures only nontypable strains were selected for the purpose of finding phages that attacked this group of strains. The attack rate in the crosscultures of such strains was low, only about 0.5%.

The 5 new phages that were selected proved to be useful for differentiation of strains within the group of hitherto nontypable strains. Together with another new phage, the effect of which was visible on primary culture from an ear discharge, the 6 phages were able to type 73% of 158 nontypable strains tested. One phage proved to be related to phages 80 and 81, but the other 5 seem to be quite specific. They attacked only a few of those strains that characterize the different standard phages, and, generally only one of them attacked the nontypable strains tested, thus giving 5 characteristic

"types." Provisionally the phages have been called A, B, C, D, E and F. A, C, and D, seem to be related to the Group III phages, F to 80/81, B to other Group I phages and E to none of the others.

Summary and conclusions. The isolation of 7 new staphylococcal phages is described; 5 of them appear to be quite distinct and specific and the other 2 are related to the 80/81 phages. With the 5 new and specific phages and one of the others it was possible to type 73% of 158 strains which were recently isolated from infected lesions, nontypable with the conventional set of phages and most of them resistant to the most frequently used antibiotics. These new phages may, there-

fore, prove useful for phage typing of pathogenic staphylococci.

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Comparative Activity of Various Penicillins Against Penicillinase-Producing and Nonpenicillinase-Producing Staphylococci.* (26244)

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The isolation of 6-aminopenicillanic acid (APA) from penicillin fermentations was heralded as an important discovery making possible the synthesis of many new penicillin derivatives which could not be prepared by direct biosynthesis.(1). The introduction, within less than a year, of two new, therapeutically useful semisynthetic penicillins(2, 3) with enhanced activity against penicillin-resistant staphylococci and the likelihood of the development fairly soon of other such penicillins with substantially or radically different antibacterial and other properties, has already resulted from this discovery. Comparisons of the true therapeutic value against human infections of the new semisynthetic

penicillins with those already available, or which can be prepared by direct fermentation methods, is extremely arduous and some penicillins which have similar *in vitro* and pharmacologic properties, may be therapeutically indistinguishable. Nevertheless, any valid *in vitro* or *in vivo* comparisons may be useful in the long run in correlating chemical structure with therapeutic activity for the ultimate purpose of synthesizing "tailor-made" drugs. The results of comparisons of the absorption and excretion of the first of the semisynthetic penicillins with others prepared by fermentation(4,5), and of their *in vitro* activity against common bacterial pathogens (6), have already been reported from this laboratory. This paper deals with more detailed comparisons of the antistaphylococcal activity of a number of penicillins, including the two semisynthetic ones that were recently introduced.

Materials and methods. The strains of staphylococci used in this study were chosen from among recent isolates from infected le-

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† The technical assistance of Clare Wilcox, Joan Yarrows, and Mildred W. Barnes is gratefully acknowledged; Dr. Bosko Postic also rendered assistance in part of this study. Dr. Wallmark's present address is: State Bacteriological Laboratory, Stockholm, Sweden.

TABLE I. Penicillins Used in This Study.

Penicillins	Designation	Supplier
Benzyl	G	Squibb
Phenoxyethyl	V	Lilly
Phenoxyethyl (phenethicillin)*	DL	Bristol (P152) Pfizer (PA228)
Phenylmercaptomethyl	PM	Lilly
Phenoxyisopropyl	PIP	Lilly
2,6-Dimethoxyphenyl	DMB	Bristol
4 Amino, 4 carboxy, n-butyl (Synnematin B)	SB	MDHL†

* Each of these preparations consisted of approximately 65% D and 35% L isomer; each of the individual isomers were also supplied by Bristol Laboratories.

† Michigan State Dept. of Health Lab.

sions, except the FDA strain 209P which was usually included as a control; all of them were coagulase positive and had been selected as subcultures of single isolated colonies. All of these strains had been phage-typed with the standard set of phages in current use(7) plus a few others and they had been tested for susceptibility to the various antibiotics in common use. Those employed in these tests with the various penicillins represented a wide variety of patterns of phage and antibiotic susceptibility. The tests for susceptibility to the penicillins were carried out chiefly on the surface of plates of heart infusion agar in which 2-fold dilutions of the antibiotics were incorporated, the highest concentrations used being either 100 or 200 $\mu\text{g}/\text{ml}$. In most instances they were inoculated with aid of the inocula-replicating apparatus of Steers, Foltz and Graves(8), but in some comparative tests the plates were inoculated with drops from a Pasteur pipette. In either case, the inoculum, when an undiluted overnight culture was used, contained 1.8×10^6 viable units. Additional tests were done in brain heart infusion broth containing 2-fold dilutions of the antibiotics and equal volumes of appropriate dilutions of an overnight broth culture. Unless otherwise noted, the tests were read after incubation for 20-24 hours at 37°C . Penicillinase production was demonstrated either by the cylinder-diffusion method of Wallmark (9) or by the modified Gots method generally employed in this laboratory(10). The various penicillins studied, the symbols used

here to designate them (some have been given other designations by other workers), and their suppliers are listed in Table I. Counts of viable units or organisms were made from pour plates of 10-fold dilutions of the cultures in agar with the addition of a large excess of penicillinase (Neutrapen, SchenLabs) to cultures containing penicillin.

Results. Comparative activity of various penicillins. Table II shows a comparison of the activity of the different penicillins against a number of strains of penicillinase-producing and nonpenicillinase-producing staphylococci tested with the inocula-replicating apparatus. The large inoculum represents the undiluted culture. Each number represents the ratio of the geometric mean of the minimum inhibiting concentration (MIC) of the particular penicillin to that of penicillin G for all the strains tested. It is seen that G, V and PM differ only slightly in their activity against both the producers and non-producers of penicillinase, regardless of whether the large or the small inoculum was used; the action of other penicillins varied with one or the other or both of these factors. The phenethicillins (D, L and DL) and also PIP and DMB, but particularly the latter, were all much more active than G, V and PM against the large inoculum of penicillinase-producers, but all

TABLE II. Relative Activity of Various Penicillins as Compared with Penicillin G.

Penicillin	Penicillinase producers*		Non-penicillinase producers†	
	$10^6/\text{ml}$	$10^2/\text{ml}$	$10^6/\text{ml}$	$10^2/\text{ml}$
G‡	1	1	1	1
V	0.7	0.5	1.3	1.3
PM	1.0	0.7	0.6	0.6
PIP	0.12	1.7	2.0	20.
D	0.15	1.0	4.9	5.2
L	0.08	0.5	1.3	1.4
DL	0.25	0.8	1.3	1.5
DMB	0.02	5.7	15.	80.
SB	1.6	104.	80.	480.

* Based on 36 strains of *Staph. aureus* except the values for D and L which were based on 14 other strains; for the latter the relative activity of DL was 0.08 (for $10^6/\text{ml}$) and 0.4 (for $10^2/\text{ml}$).

‡ Based on 20 non-penicillinase producing strains.

† The numbers represent the ratio of MIC of the various penicillins to that of G; the mean MIC's of G for the penicillinase producers were 100 and 1.6 $\mu\text{g}/\text{ml}$ for the large and small inocula, respectively, and those for the non-penicillinase producers were 0.03 and 0.02 $\mu\text{g}/\text{ml}$.

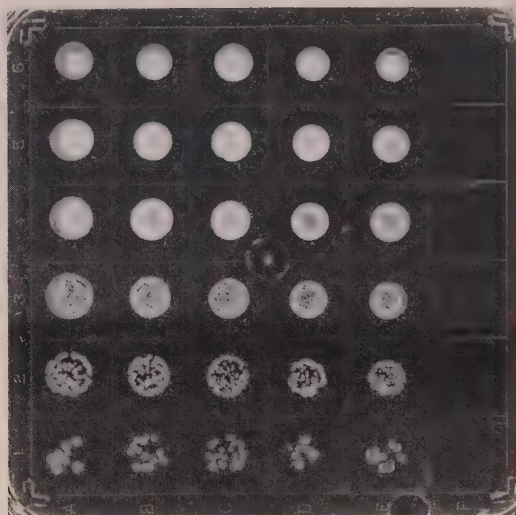


FIG. 1. Control plate (penicillin-free agar) in replica-inoculating method of sensitivity testing. The 5 strains (and their phage types) were, from left to right: No. 1 (80/81/KS6); 20 (?); 155 (81/42E); 157 (79); and 209P (42D). The log of the number of viable units in each horizontal row is shown on the left. The size of the inner, agar-covered surface of the plate is $3\frac{1}{2}$ in. square.

of them except DMB were similar in activity against the small inoculum of the same strains. DMB was considerably less active than the others against the small inoculum of these penicillinase producers. SB was almost as active as G against the large inoculum of penicillinase producers but was very much less active against the small inoculum. Against the non-penicillinase producers, L and DL were similar in activity to G, V and PM regardless of the size of the inoculum. D, DMB and SB showed relatively decreasing activity against those strains with both inoculum sizes as compared with the others, whereas PIP was only slightly less active than G against the large inoculum but much less active against the small number of organisms.

Effect of inoculum size. This was studied in greater detail and by several methods. In the first place it was shown that the inocula-replicating apparatus was admirably suited for this purpose. Figure 1 shows a control plate of penicillin-free agar inoculated with 6 serial 10-fold dilutions of 5 strains of *Staph. aureus* chosen because of the differences in the degree of their susceptibility to

penicillin G. The inocula in the top row consisted of $1-5 \times 10^6$ organisms, those in the bottom row yielded growths of 10-30 readily discernible colonies. The last plates inoculated after completion of the tests with all the penicillins contained subinhibitory concentrations of the antibiotics and were indistinguishable from that shown in Fig. 1.

The results of all these tests are represented graphically in Figure 2. It is seen that, in general, there was a progressive drop in the MIC with progressive dilutions of the cultures, the rate of decline in MIC being greatest for the most resistant strain (No. 1) and least for the most sensitive ones, namely Nos. 155 and 209P. This was true for all of the penicillins except DMB, which inhibited the largest inocula of all 5 strains in the same concentrations, namely $3.1 \mu\text{g/ml}$, and which required one-half of that concentration to inhibit the smallest inocula.

The same 5 strains were also tested for the effect of inoculum size on the bacteriostatic and bactericidal activity of these 7 penicillins in the broth-dilution method. In these tests 4 different dilutions (10^{-1} , 10^{-2} , 10^{-4} and 10^{-6} , final) of each culture were used and readings of visible growth were recorded after incubation for 24 hr and again at 48 hr; subcultures were made to penicillin-free agar at each of these times. Table III shows the bacteriostatic (S) and bactericidal (C) concentrations of the various penicillins for the different inocula, the former representing absence

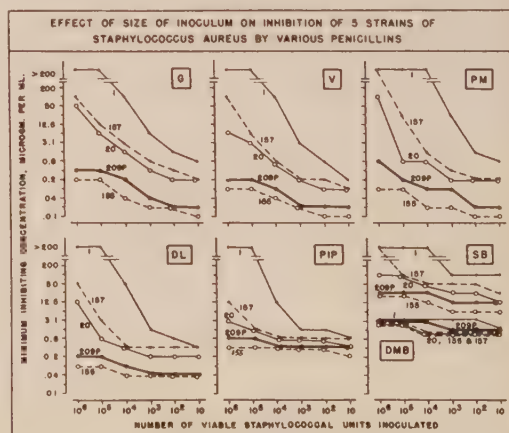


FIG. 2. The 5 strains are the same as those represented in Fig. 1.

TABLE III. Bacteriostatic and Bactericidal Action of 7 Penicillins on *S. aureus*: Effect of Size of Inoculum.

Strain	Inoculum : Viable units/ml	Type of penicillin													
		G		V		PM		DL		PIP		DMB		SB	
		S	C	S	C	S	C	S	C	S	C	S	C	S	C
		Minimum bacteriostatic (S) and bactericidal (C) concentrations,† $\mu\text{g/ml}$													
		micrograms per milliliter													
1	2×10^7	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200
	2×10^6	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200
	2×10^4	50	>200	100	>200	>200	25	>200	>200	12.5	>200	1.6	25	>200	>200
	2×10^2	1.6*	1.6*	0.8	0.8*	0.4	0.4*	0.8	0.8*	1.6	3.1	1.6	3.1*	100	100
20	4×10^7	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	1.6	>100	>200	>200
	4×10^6	>200	>200	>200	>200	>200	>200	>200	>200	25	>200	1.6	6.3	>200	>200
	4×10^4	0.8	25	0.8	25*	1.6	50	0.8	25*	1.6	12.5*	1.6	6.3	50	>200
	4×10^2	0.4	0.4	0.1	0.1*	0.1	0.1	0.2	0.2*	0.8	0.8	1.6	1.6	25	50
155	5×10^7	0.04	0.8	>200	25	>200	6.3	>200	100	>200	50	3.1	>100	12.5	>200
	5×10^6	0.02	0.2	0.02	0.2	0.02	0.1	0.04	0.1*	0.4	1.6*	1.6	3.1	12.5	25*
	5×10^4	0.02	0.02*	0.02	0.04	<0.01	0.1	0.02	0.04*	0.2	0.4	0.8	1.6	6.3	12.5
	5×10^2	<0.01	0.1	0.02	0.02	<0.01	<0.01	0.02	0.02	0.2	0.2	0.8	0.8	6.3	6.3
157	2×10^7	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200	1.6*	>100	>200	>200
	2×10^6	>200	>200	>200	>200	>200	>200	>200	>200	25	>200	1.6	3.1*	>200	>200
	2×10^4	6.3	200	3.1	200	1.6	25	1.6	200	0.8	100	1.6	3.1	100	200
	2×10^2	0.4	0.4	0.2	0.2	0.2	0.4	0.2	0.2*	0.8	1.6*	0.8	1.6	50	50
209P	10^7	≥ 12.5	6.3	≥ 12.5	≥ 12.5	≥ 50	25	≥ 12.5	≥ 12.5	≥ 100	≥ 100	>100	>100	25	50*
	10^6	0.1	0.2*	0.04	≥ 12.5	0.04	0.2*	0.1	3.1	0.4	≥ 100	3.1	3.1	25	50
	10^4	0.04	0.1	0.02	0.1	0.02	0.04	0.04	0.1	0.4	0.8	3.1	3.1*	12.5	25
	10^2	0.02	0.1	0.02	0.04	0.02	0.04	0.04	0.04	0.4	0.4	1.6	1.6	12.5	12.5

* Irregular; no growth from all but 1 or 2 tubes with higher concentrations.

† Bacteriostatic concentrations are those with no visible growth in broth at 24 hr; bactericidal concentrations indicate no growth on subculture of 48-hr broth culture to agar.

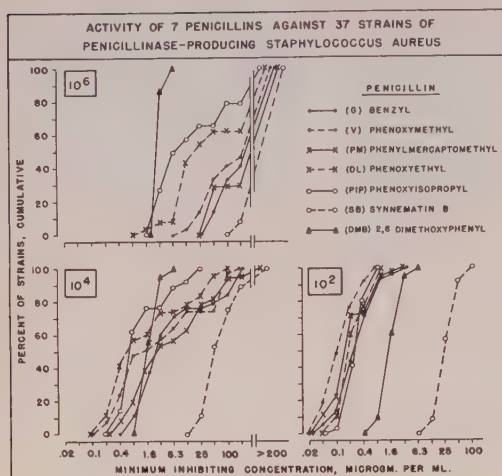


FIG. 3.

of visible growth in the broth at 24 hr, and the latter absence of growth from the subculture on agar made from the broth at 48 hr. In these tests, none of the penicillins, including DMB, were bactericidal for the largest (10^7) inocula, otherwise the results were roughly comparable to those obtained in the agar dilution tests mechanically inoculated.

Another series of tests were done with 37 penicillinase-producing staphylococci by the inocula replicating method, using the cultures undiluted and also diluted 1/100 and 1/10,000, representing inocula of approximately $(1.5 \times) 10^6$, 10^4 and 10^2 viable staphylococcal units, respectively. A comparison of the results obtained with each inoculum is shown in Fig. 3. With the largest of these inocula (10^6) there were wide variations in the MIC of each antibiotic for the different strains, except in the case of DMB which was the most active and was essentially equally active against all the strains, and also in the case of SB which was essentially inactive against nearly all the strains in the highest concentration used. The order of activity of these penicillins against the 10^6 inoculum was: DMB > PIP > DL > V > G > PM > SB. Qualitatively similar but quantitatively less marked differences were noted with the intermediate inocula (10^4). The smallest inocula (10^2) were inhibited by all the penicillins except DMB and SB in nearly the same concentrations, and there were much less differ-

ences in the MIC of the individual penicillins for the different strains. The most striking effects were shown by G and PM with a tendency for decreasing effects with V, DL, PIP, and SB, in that order. There was only a minimum effect of inoculum size on the activity of DMB. Additional tests were done with 48 other strains of penicillinase producing staphylococci, using inocula of 10^6 , 10^5 , 10^4 and 10^3 organisms and serial dilutions of G, V, D, L, DL and PM. The curves representing the results of these tests were very similar to the corresponding ones in Figure 3. In these tests D was usually $\frac{1}{4}$ as active as L against nearly all strains and DL was about as active as L when the inocula were 10^6 or 10^5 ; with the smaller inocula, D was generally about $\frac{1}{2}$ as active as L and DL.

Similar tests were carried out with 28 strains of nonpenicillinase producing staphylococci, using the cultures undiluted (10^6 organisms) and diluted 1/1000 (about 10^3 viable units). In these tests, the large inocula of nearly all the strains were inhibited by G, V, L and DL in concentrations of 0.02 and 0.04 $\mu\text{g}/\text{ml}$, by $\frac{1}{2}$ of these concentrations of PM, and by 4 times these concentrations of D. The smaller inocula were inhibited by $\frac{1}{2}$ of the corresponding concentrations in almost every instance. Only a few of these strains were tested with PIP, DMB and SB; all were about equally susceptible to each of these penicillins and the results were quantitatively similar to those shown for strains 155 and 209P in Figure 2, there being only a 2-fold difference between the MIC of the larger and the smaller inocula for each of these penicillins.

Viable counts. Eight separate tests were done with 2 strains of non-penicillinase producing staphylococci, namely 209P and No. 81 (phage pattern 29/80) in broth. Tests were done simultaneously with penicillins G, V, DL and PM at a final concentration of 1.0 $\mu\text{g}/\text{ml}$, and a penicillin-free culture was included in each instance. The starting inocula varied from 7×10^4 to 3×10^6 organisms per ml in the different experiments, aliquots being removed and viable counts made (with addition of penicillinase in excess to all

penicillin-containing cultures) at frequent intervals during the first 12 hours, and then at 22-24, 48, 72 and 96 hours. Control tubes without penicillin all showed some multiplication at 3 hours and full growth ($4-14 \times 10^8$ ml) by 8 or 12 hours with a slow decline at 24 hours and thereafter. In the penicillin-containing tubes, the counts were nearly always the same at 3 hrs as at the start; after that there was a steady drop to between 1.0 and 0.1% of the original number at 12 hr, and to between 0.01 and 0.001% at 24 hr. In most instances there were still some viable organisms (0-100) at 48 hr. All the penicillins tested behaved alike quantitatively in nearly all experiments except that PM was more rapidly bactericidal in most instances and was the only one that completely sterilized one of the cultures at 22 hours; in that experiment the starting inoculum was 7×10^4 viable units/ml. In simultaneous comparisons of 5 and 0.5 $\mu\text{g/ml}$ in one experiment and of 6.3 and 0.4 $\mu\text{g/ml}$ in another, no difference was observed in the rate of drop off of the viable counts; indeed in one of these experiments the counts dropped more rapidly during the first 24 hours in the tubes containing 0.4 $\mu\text{g/ml}$ than in those with 6.3 $\mu\text{g/ml}$, but the counts were similar at 48 hours and there were no viable organisms at 72 hours in that test. In all these tests, the phage pattern of the final growth was checked to rule out any contamination.

A penicillinase-producing, nonphage typable staphylococcus, No. 236 (M.I.C. >100 $\mu\text{g/ml}$), was used in 7 experiments starting with $5.5-6 \times 10^5$ viable units per ml. Penicillins G and DL were used in every test, V and MP were also used in 4 of them, and D and L were used, in addition, in 2 of the latter; these were added to the cultures in concentrations of 0.4, 6.3 and 100 $\mu\text{g/ml}$. The control culture without penicillin behaved as in the previous experiments. In nearly all of the cultures containing penicillin (except in 1 of 2 tests with 0.4 $\mu\text{g/ml}$ of G in which multiplication already occurred at 6 hours) there was a reduction in the number of viable units to between 10% and 1% in the first 12 hours of incubation. After that time, multiplication was resumed in every instance ex-

cept in 1 of 3 which contained 100 $\mu\text{g/ml}$ of DL and in which the number of organisms continued to decline until none were grown at 72 hours. Regrowth to about the original number generally occurred by 48 hours and to control numbers by 72 hours except in cultures containing L or DL which were not fully grown until 96 hours; in 2 instances, however, the number of organisms in the presence of 0.4 and 6.3 $\mu\text{g/ml}$ of penicillin G equalled that of the control at 24 hours.

These experiments show that the action of penicillins G, V, PM and DL was generally very similar against both the producers and nonproducers of penicillinase with the inocula and the concentrations of the drugs used, except that the growth of the penicillinase producer was somewhat faster in the presence of G and was longer delayed with DL than with V or PM. The question thus raised is whether the more rapid growth in the presence of G depends on its more rapid inactivation.

Inactivation by Staphylococcal Penicillinase. Several experiments were carried out with 3 penicillinase-producing strains of *Staph. aureus*; penicillins G and DL were used each time and penicillin V in half of the experiments. Preliminary observations were also made with PIP and DMB. It was shown, first of all that staphylococcal penicillinase present in fully grown broth cultures was regularly and completely removed by Seitz filtration, but that each of the penicillins was quantitatively recovered after similar filtration. In 5 experiments in which fully grown (overnight) cultures of Strain 35 (phage type 29/80) were incubated with 200 μg of penicillin per ml, from 85 to 98% of the penicillin was inactivated in 5 minutes, and all of the penicillin was inactivated at 10 minutes. Penicillins G, V and DL behaved more or less similarly in all these experiments and the differences observed were minor and irregular. In one experiment with Strain 214 (type 80/81/KS6), the organism grew poorly and neither G nor DL were inactivated by the light, 18 hr growth of this organism in 5 minutes, but at 15 minutes 25% of the G and 12.5% of DL was still active. In 3 experiments with Strain 221 (type 53), varying

and similar amounts (12.5-50%) of G and DL (200 μ g/ml was used) were still active after exposure to a fully grown culture for 5 min but all of these penicillins were inactivated by 10 min incubation with this culture. In single experiments, PIP was inactivated more slowly than G, 6% of the former still being active after 20 minutes; DMB on the other hand showed only slight (less than 10%) inactivation after several hours incubation with this culture. Slight activity of penicillinase producing staphylococci against DMB was also demonstrated in penicillin-containing agar seeded with *Staph. 209P* in the Wallmark cylinder-plate method(9) and in similar agar plates seeded with *Sarcina lutea* in the modified Gots test(10). The studies on penicillinase inactivation are being continued in this laboratory by Dr. Leon D. Sabbath.

Discussion. The data presented show considerable differences among the various penicillins tested, with respect to their activity against pathogenic strains of *Staph. aureus*. Three of them, viz. G, V and PM differed only slightly in their *in vitro* activity against both producers and nonproducers of penicillinase regardless of whether a large or small inoculum was used. The phenethicillins (D, L and DL) and PIP were considerably more active than either G, V or PM against penicillinase producers when the inoculum was large but not when it was small. Against nonpenicillinase producing staphylococci, PIP and D were less active, and L and DL about equally active, as compared with G, V and PM regardless of the size of the inoculum. DMB behaved differently than the others, it was much more active against large inocula of penicillinase producers but much less active against small inocula of the same organisms and against nonpenicillinase producers regardless of the inoculum size. Finally, SB, which was nearly as active as G against the large inoculum of the penicillinase producers,

was also very much less active against the small inocula of these strains and against nonpenicillinase producers irrespective of inoculum size.

The effect of the size of the inoculum on the action of penicillins has been recognized by many workers(11-13) but its possible clinical significance and that of the relatively minor differences in the susceptibility of some of these penicillins to degradation by penicillinase such as has been shown with DL(2) have been a matter of dispute(12-15). In the present study, the greater resistance to *Bacillus cereus* penicillinase of DL as compared with G and V(2) was not confirmed insofar as staphylococcal penicillinase is concerned. The similar activity of DMB against producers and nonproducers of penicillinase and hence its much greater activity than any of the other penicillins against the former and its much poorer activity against the latter, makes this antibiotic potentially highly useful against penicillin-resistant staphylococcal infections but a much less desirable one for the treatment of infections due to nonpenicillinase producers. Its effectiveness against penicillin resistant staphylococcal infections has already been attested in a number of recent reports.[†] Since DMB and G are both absorbed and excreted in about the same manner after parenteral injection, the former would certainly not be the drug of choice against infections with nonpenicillinase producing staphylococci.

Summary. Several penicillins were tested *in vitro* for their activity against penicillinase-producing and nonpenicillinase-producing strains of *Staphylococcus aureus*, with particular reference to the effect of the size of the inoculum. Marked differences were demonstrated, depending on the type of penicillin, the strain of staphylococcus and the size of the inoculum.

[†] Several of these appeared in the *Brit. M. J.* for Sept. 3, *Lancet* for Sept. 10, and others were presented at a Conference in Syracuse, N. Y. Sept. 7, 1960 and will be published in monograph form by the Syracuse University Press.

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Latent Viral Infection of Cells in Tissue Culture. VIII. Morphological Observations of Psittacosis Virus in L Cells.* (26245)

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With psittacosis virus, a latent infection can be established in cultures of L cells by maintaining them in a balanced salt solution (1) or a synthetic medium deficient in an amino acid(2) for 2 days prior to infection. When the virus infects these cells, it enters a latent phase which will persist in the cells for several days, during which time the addition of a medium consisting of water-soluble vitamins and amino acids will cause the virus to multiply. The objective of this study was to examine infected cells during this period of latency and early in the resumption of viral propagation in an attempt to determine whether any morphological structures occur which might be characteristic of this latent infection and how many cells are infected at various intervals of time after inoculation with virus.

Materials and methods. 1. *Media:* A lactalbumin-horse serum-yeast extract (LHYE) medium(1), Earle's balanced salt solution (BSS), and a synthetic medium (CM) consisting of salts, water-soluble vitamins and amino acids(2), and this synthetic medium minus its content of phenylalanine (PDM) were employed. 2. *Virus and viral titra-*

tions: The 6BC 3cl strain of psittacosis virus, obtained from Dr. J. W. Moulder and maintained in the laboratory by allantoic fluid passage, was diluted 1/1000 in BSS or PDM and inoculated into monolayer cultures of L cells maintained in BSS or PDM. Fluids were changed daily and titrated by the single dilution method of Golub as modified by Dougherty *et al.*(3). On the appearance of a cytopathic effect, each cell layer was scraped off and the suspension was shaken with 0.5 g of 0.9 mm-diameter glass beads. Supernatant fluids were removed from each suspension and were dispensed in ampules and preserved at -70°C for inoculation of tissue cultures. All virus titrations were carried out by the modified titration method(3). 3. *Experimental protocol:* Petri dishes containing cover slips were seeded with L cells in LHYE medium and placed in a CO_2 incubator at 37°C . When a monolayer of cells had been established on the cover slips, the cell sheet was rinsed 3 times with warm BSS. L cells were maintained for 48 hours in BSS or PDM with daily fluid changes, the cultures were inoculated with a 1/1000 dilution of stock virus containing $10^{3.5}$ LD₅₀ for infection, and culture fluids and lysed cells were tested for virus content as indicated. 4. *Light microscopy techniques:* a) Methyl alcohol-fixed cells on cover slips were stained with May-

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Grunwald-Giemsa (MGG) stain as described by Jacobsen(4); b) Feulgen staining, as described by Chargaff and Davidson(5), was performed on methyl alcohol-fixed cells. Slides were counterstained with 0.1% light green to increase the contrast between Feulgen-negative and Feulgen-positive structures; c) Acridine orange staining, as described by Armstrong and Niven, was employed(6). A standard Zeiss microscope with a mercury lamp was used for observation. 5. *Electron microscopy*: Infected cells were sedimented by centrifugation and fixed at 0.5°C for 30 minutes in veronal acetate-buffered 1% osmium tetroxide containing 0.22 molar sucrose (7). Pellet fragments were dehydrated in ethyl alcohol and embedded in n-butyl-methacrylate. Sections were stained with lead hydroxide(8) and sandwiched with formvar (9). Electron micrographs were made with a Siemens Elmiskop 1 electron microscope.

Results. When L cells maintained for 2 days in each medium were exposed to the virus in each of the 3 different media, *i.e.*, LHYE, BSS, and PDM, for 8 hours, the virus was found to disappear from the culture fluids and to be detectable in small amounts in the cells after washing and lysis. However, tests of cells 24 hours after infection failed to reveal any infectious virus in the cells maintained in BSS or PDM, though virus reappeared within 24 hours after addition of the synthetic medium (CM) containing a complete complement of water-soluble vitamins and amino acids as has been previously reported(1,2). These results indicate that the virus infects cells maintained in BSS or PDM and enters a latent phase until cell nutrition is altered by supplying the missing vitamins and/or amino acids.

To determine the number of cells that were infected when exposed to virus, cover slips were removed from each preparation and cells were stained and examined for characteristic virus inclusions 24 hours after infection in LHYE and 24 hours after addition of the CM medium to those cells that had been infected in BSS or PDM. In all 3 cases, approximately 10% of the cells were found to contain characteristic virus inclusions.

An attempt was made to increase the num-

ber of cells infected (a) by reexposing the cells maintained in each of the media to fresh inocula of virus at 2-hour intervals during an 8-hour period at 37°C in which the cells were gently agitated, and (b) by reexposing the cells to fresh inocula of virus at 8-hour intervals for 24 hours at 4°C. With the LHYE medium, after 24 hours of incubation at 37°C and with BSS following the addition of the CM medium and 24 hours of incubation at 37°C, again only about 10% of the cells were found to be infected when examined in stained preparations. Thus agitation or repeated exposure of cells to the virus did not increase the proportion of cells susceptible to infection.

Morphological changes in infected cells were then studied, using the May-Grunwald-Giemsa and Feulgen stains as described. With the LHYE medium, inclusion bodies were first visible at 16 hours after infection and these became more intensely Feulgen-positive at 22 hours as the individual elementary bodies became visible. Such cells also revealed the characteristic collections of psittacosis elementary bodies when examined with the electron microscope. By 72 hours of incubation, 70% of the cells were infected, and when overt signs of cell destruction were visible at 96 hours, almost all cells contained inclusion bodies. When similar examinations of infected cells maintained in the BSS or PDM were made, no inclusion bodies were seen during the period of latent infection with light or electron microscopy. Within 22 hours after these cells were fed with the CM medium, inclusion bodies appeared within the cells and the infection spread to involve all the cells with destruction of the culture.

With the acridine orange stain, no changes were visible in infected cells until 24 hours after infection at which time discrete, round or oval orange-red masses appeared within the cytoplasm. These masses were the same as the inclusion bodies seen in the MGG stain, for when the acridine orange stain was removed and the cells were restained with MGG, the orange-red bodies appeared as typical viral inclusions. When the infected cells were treated with ribonuclease, the inclusion bodies underwent a striking change,

appearing as deep green homogeneous masses, whereas treatment with desoxyribonuclease resulted in an intensification of their red fluorescence. These results suggest the presence of DNA and RNA in the virus inclusions as indicated by the studies of Armstrong and Niven(6).

Discussion. Infection of L cells with psittacosis virus in a variety of media, including LHYE, CM or BSS, reveals that virus penetrates the cells in BSS alone and thus apparently only salts and glucose are essential for this step and the subsequent step of development of a latent phase of virus infection. No viral structure characteristic of this latent infection could be visualized by light or electronmicroscopy in the cells examined, but only 10% of these were shown to be infected, and examination of much larger numbers of cells might have revealed some virus structures such as the early virus forms described by Litwin in infections with feline pneumonitis virus(10). Subsequent virus multiplication will not occur in these cells in absence of water-soluble vitamins and certain amino acids(2). However, under the conditions of these experiments, only 10% of the cells could be infected at any one time with virus grown in the same cells, though with continued incubation and release of virus from the infected cells, infection spread to all cells and the culture was destroyed. This occurred equally well in a synthetic medium (CM) containing only salts, glucose, glutamine, water-soluble vitamins and amino acids or LHYE. Since all the cells eventually became susceptible to infection over a period of 96 hours, a cyclical change in cell susceptibility is indicated. It might be thought that this would be related to the mitotic cycle, but the CM medium does not support cell multiplication so that this possibility is eliminated. Some physiological state of the cell appears most likely to be the determining factor of its susceptibility to infection, and over a period of 96 hours the virus infects and destroys all cells as conditions compatible with

infection develop in segments of the cell population. A similar situation was observed by Officer(11) in infection of Chang's strain of human liver cells with the 6BC strain of psittacosis virus where only 2% of the cells could be infected at any one time.

There is a possibility that an asynchronous infection might have been established in these cultures in which only part of the infected cells were at a stage, at 24 hours following addition of virus, in which visible viral inclusions were present, but morphological studies on growth of this and related viruses have provided no evidence for asynchronous infection of cells(10,12).

The histochemical studies indicate that the intracellular inclusion bodies of psittacosis virus contain RNA and DNA in confirmation of other observations(13,14).

Summary. With the 6BC strain of psittacosis virus only 10% of L cells could be infected *in vitro* in several culture media, even with repeated exposure to virus over a period of 8 hours at 37°C or 24 hours at 4°C. However, all cells became susceptible to infection during a period of 96 hours at 37°C, suggesting a sequential change in susceptibility. Using the balanced salt solution of Earle or a synthetic medium deficient in phenylalanine, the virus infected the cells and entered a latent phase as well as it did with a complex horse serum-lactalbumin hydrolysate-yeast extract medium, but virus growth did not occur until the missing amino acid or acids were supplied. No structures characteristic of virus were visible in these cells during the phase of latent infection when examined with the light or electron microscope.[†] Histochemical studies of the growth of virus in L cells revealed that the virus inclusion body contained RNA and DNA.

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Relative Effectiveness of Phenothiazine Tranquilizing Drugs Causing Release of MSH.*† (26246)

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(Introduced by Ivor Cornman)

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The ataraxic drug meprobamate (Miltown, Equanil) has been found to cause darkening of the light-adapted frog. This reaction does not occur in the hypophysectomized animal (1). Chlorpromazine (Thorazine), methoxypropazine (Tentone), and reserpine also have been found to cause melanophore dispersion in the normal frog and in the sand dab, *Lophosetta maculata*. On the other hand, azacyclonol (Frenquel) and oxanamide (Quiactin) have been found to be ineffective (2).

This paper is a report of the influence of 10 phenothiazine derivatives now used in medical practice on the pituitary melanocyte system of the frog, *Rana pipiens*. Marked differences in milligram potency of the drugs causing release of melanocyte stimulating hormone (MSH) from the hypophysis were observed.

Methods. Three hundred and thirty-one medium-sized *Rana pipiens* of both sexes were used in determination of the minimal effective doses (MED) of the drugs. The effects on 10 hypophysectomized frogs were also observed. Frogs were weighed and

placed singly in 1000 ml beakers containing 200 ml of tap water. To provide a white background, beakers were wrapped with white cloth. Beakers containing the frogs were placed in white enamel pans under continual illumination from a pair of 4 foot, 40 watt fluorescent tubes mounted in a fixture 20 inches above the pans. Frogs were permitted to light-adapt overnight prior to injection. Equal mg% doses (mg of drug per 100 g of frog) of a drug were injected (0.1-1.3 ml) intraperitoneally into a series of 3 to 5 light-adapted frogs. Doses ranged from 0.01 to 8.0 mg%. The extent of melanophore dispersion was recorded from microscopic observation of the web of the foot at 2-5 hour intervals for 36 hours following injection. The melanophore index of Hogben and Slome(3) was used. Twenty-seven control frogs injected with carrier solutions and 5 uninjected controls were also run.

The mean melanophore response to each dose was calculated by averaging the single maximal responses of all frogs(3-11) injected with that dose. The MED was defined as the minimal dose eliciting a mean melanophore response of 3.0 on the Hogben and Slome index and was obtained by interpolating from a dose-response curve.

Hypophysectomy was performed according to the procedure of Hogben(4) with additional use of an ice bath to reduce blood flow. Complete removal of the hypophysis

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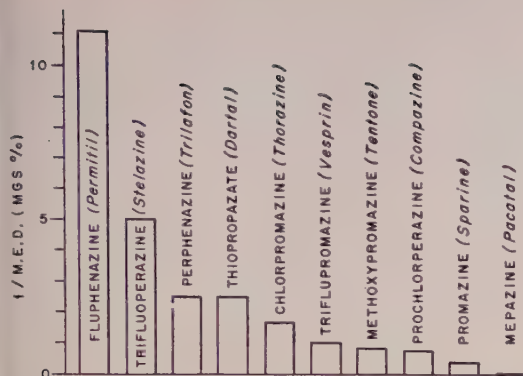


FIG. 1. Potency of phenothiazine tranquilizing drugs in causing activation of melanophores by MSH.

was indicated by punctate melanophores after one to 3 days on an illuminated, black cloth background following the operation.

The following drugs were used: methoxy-promazine (Tentone), chlorpromazine (Thorazine), triflupromazine (Vesprin), trifluoperazine (Stelazine), prochlorperazine (Compazine), thiopropazate (Dartal), fluphenazine (Permitil), promazine (Sparine), mepazine (Pacatal), and perphenazine (Trilafon). Because of the relative insolubility of Trilafon and Pacatal, these drugs were suspended in an amphibian Ringer's solution containing 25% ethanol. All other drugs were dissolved in amphibian Ringer's.

Results and discussion. The reciprocal of the MED was used to compare the relative effectiveness of the various drugs (Fig. 1). All drugs caused melanophore dispersion in normal frogs with the exception of Pacatal. Carrier solutions did not cause darkening.

All drugs tested in hypophysectomized animals were without effect. These included Permitil, Stelazine, Dartal, Thorazine, Tentone and the non phenothiazine ataraxics meprobamate and reserpine.

Since the ataraxic drugs employed cause no dispersion of melanophore pigment in hypophysectomized frogs, it is concluded that these drugs bring about release of MSH by direct action on the pituitary gland or indirectly *via* brain centers. The possibility is recognized that the effect may be one of potentiation of MSH instead of release of active hormone. Experimental evidence indicates this not to be the case.

Permitil (1 mg%) does not delay paling when injected into frogs shortly after hypophysectomy while circulating MSH is presumably still present; nor did Permitil produce darkening in animals injected with sub-threshold doses of Pituitrin (Parke Davis).

There is evidence reported in the literature that the release of certain pituitary hormones is chronically inhibited by the nervous system and that drugs may effect their release by counteracting this inhibition. It was suggested several years ago (5) that melanophore hormone might be under inhibitory hypothalamic control in the tadpole.

The data (Fig. 1) are of interest in relation to the chemical structures of the phenothiazine drugs (Fig. 2). The classes of drugs are distinguished on the basis of substitution of a piperazinylpropyl, a dimethylaminopropyl, or a piperidylmethyl group at position 10 of the phenothiazine nucleus. Drugs within these classes are characterized by further substitutions at position 2 of the phenothiazine nucleus and at position 1 of the piperazine ring.

Relationships between various substitutions on the phenothiazine nucleus and milligram potency for pigment dispersing action are summarized as follows: 1. The most active drugs (Permitil and Stelazine) contain in trifluoromethyl group and the piperazine ring. 2. The next 2 most active derivatives (Trilafon and Dartal) contain chlorine and the piperazine ring. 3. With the exception

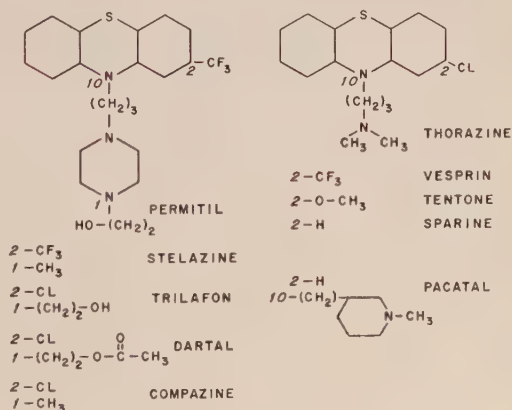


FIG. 2. Chemical structures of phenothiazine tranquilizing drugs. Substitutions are indicated at positions 1, 2 and 10.

of Compazine, the next 5 drugs, in decreasing order of milligram potency (Thorazine, Vesprin, Compazine, Tentone, and Sparine), contain the dimethylamino group in place of the piperazine ring. 4. The only ineffective drug, (Pacatal) is the only one containing the piperidine ring.

It can therefore be concluded that of the 3 substituents at position 10, the piperazinyl-propyl group is more effective than the dimethylaminopropyl group and that the piperidylmethyl group produces an ineffective drug. Trifluoromethyl is generally more effective than chlorine or other substituents at position two.

Our results show a striking parallel with the results reported by Ayd(6) comparing the milligram potency of these same drugs required for therapeutic control of a wide spectrum of psychiatric disorders in 4,000 ambulatory and hospital patients. The relative ranking of the 10 drugs for milligram potency was identical in both studies with the exception of Thorazine, which was less effective than Compazine and Vesprin in Ayd's study, and of Compazine, which Ayd found to be more effective than Vesprin and Tentone.

It may be concluded that the relative milligram potencies of phenothiazines are similar for pigment dispersion in the frog melanocyte and for therapeutic control of human psychiatric illness.

Summary. Nine phenothiazine ataraxic drugs were found to cause melanophore dispersion in the intact frog; one drug was shown to be ineffective. All drugs tested in hypophysectomized animals were without effect. The drugs, therefore, are effecting the release of MSH by direct action on the hypophysis or indirectly *via* brain centers. Minimal effective doses of the drugs were correlated with substitutions on the phenothiazine nucleus. A parallel with milligram potency for therapeutic control of psychiatric disorders is noted.

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Direct Recordings from Chronically Implanted Electrodes on the Specialized Conducting System of the Heart.* (26247)

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This laboratory has previously described a technic for studying conduction within the heart by utilizing electrodes placed at selected sites on the endocardium under direct vision during cardiopulmonary bypass(1,2,3). Such studies, until recently, have been carried out only during acute experiments. It is now possible to implant multi-lead electrodes over the bundle of His, the Purkinje-

papillary junctions and on other areas within the heart with complete recovery of the animal from the surgical procedure. Records from these intracardiac electrodes, as well as from surface electrodes on the atria and ventricles, can be monitored through external leads over extended periods of time.

Method. Adult mongrel dogs weighing 20-28 kg were anesthetized with an intravenous injection of a 2½% solution of thiopental sodium, intubated, and placed on a respirator.

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The right chest was entered through the bed of the fifth rib, tapes were placed around the superior and inferior venae cavae and the pericardium opened widely. Sterile plastic electrodes containing 5 contacts were sutured to the epicardium of the right atrium and the right and/or left ventricles. Animals were then placed on total cardiopulmonary bypass; a heat exchanger was incorporated in the circuit to control the temperature of the dog. The right atrium was opened, the bundle of His was located with an exploring electrode(2) and a permanent 5 contact recording electrode sutured in position. The right ventricle then was opened and a similar electrode sutured in position over the Purkinje-papillary junction of the anterior papillary muscle. Sterile leads from this latter electrode were brought out through the upper end of the ventriculotomy wound which was closed with interrupted cotton sutures. Sterile leads from the electrode over the bundle of His were brought out through the right auricular appendage. The animal was taken off perfusion and the right atrium closed with a running 4-0 arterial suture. Leads from the intra- and extra-cardiac electrodes were brought out to the surface of the dog and the chest incision closed in layers. The recording electrodes consisted of fine silver wires embedded in plastic plaques. Each of the 5 wires was insulated with a coating of teflon; the leads from each electrode were placed within a polyethylene tube and the entire unit sterilized. Electrograms were monitored on an Electronics for Medicine 8-trace oscilloscope and permanent records made on photographic paper moving at 200 mm/sec. Records from electrodes on and within the heart were filtered to eliminate low frequency components; filter settings are specified for each record.

Results. Electrograms recorded on the first postoperative day and the twentieth day through electrodes located on the right atrium, over the bundle of His and on the right ventricle are shown in Fig. 1. Also shown are records of leads I, II, and III obtained 5 months after electrode implantation. Fig. 2 shows tracings from another animal obtained 5 days after operation. In this ex-

periment an additional electrode was placed on the Purkinje-papillary junction of the anterior papillary muscle of the right ventricle. Inspection of the records in both figures reveals activity recorded through the electrode on the right atrial appendage (A_1 ,A) clearly precedes the P wave of lead II and atrial activity recorded through the electrode located over the His bundle (A_2) appears during the initial part of the P wave. The time required for activity to spread from the region of the pacemaker to the vicinity of the A-V node is 20-40 msec. The deflection recorded from the bundle of His (H) appears midway in the P-R segment (Fig. 1, first day, Fig. 2) and indicates the usual subdivision of this segment into intervals associated with transmission in the A-V node and conduction in the His-Purkinje system. In Fig. 2 the electrogram recorded from Purkinje fibers at the Purkinje-papillary junction (P) is simultaneous with the beginning of the Q wave in lead II and electrical activity recorded from the papillary muscle (M) is present during this initial deflection of the electrocardiogram. The records obtained on one animal 20 days after implantation (Fig. 1) are included because they show an increase in P-R interval. The electrogram from the His bundle show that this change is due entirely to an increase in the time required for A-V transmission. There is also some change in the S-T segment and T wave of lead II; these changes did not persist, as can be seen from the electrocardiogram recorded 5 months after electrode implantation.

Discussion. Electrograms recorded directly from different parts of the specialized conducting system through chronically implanted electrodes confirm certain results of acute experiments. For example, it is apparent that atrial excitation reaches the region of the A-V node early during the P-R interval of the electrocardiogram. Nodal delay occupies the latter part of the P wave and the initial part of the P-R segment while the latter part of this interval is associated with the spread of activity through the bundle of His and the major subdivisions of the Purkinje system (4). Also, activity in ventricular muscle fibers in the anterior papillary muscle is re-

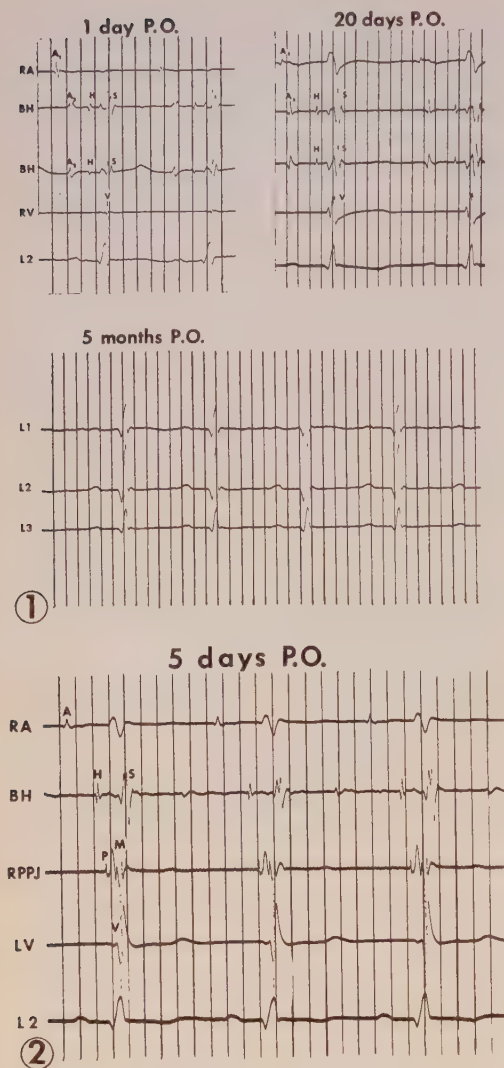


FIG. 1. Electrograms obtained 1 and 20 days following operation. Electrodes are positioned as follows: Right atrium near SA node (RA), bundle of His with 2 different filter settings (BH), right ventricle (RV) and a standard lead II electrocardiogram (L2). Components of electrograms are labeled as follows: A_1 = right atrium near SA node; A_2 = right atrium recorded at the His electrode; H = bundle of His; S = septum recorded at the His electrode, and V = right ventricle. Paper speed 200 mm/sec., time lines = 40 msec. Settings of low and high pass filters, in cps are for RA, 4/200; BH (line 2), 40/200; BH (line 3), 12/200; RV, 4/200; L2, 0.1/200. Polarity of His electrogram reversed in records obtained at 20 days; gain increased for upper trace. Leads I, II, III taken 5 mo following operation.

FIG. 2. Electrograms obtained 5 days following operation. Electrodes are positioned as follows: right atrium near SA node (RA), bundle of His (BH), Purkinje-papillary junction of right ante-

rior papillary muscle (RPPJ), left ventricle (LV), and lead II of a standard electrocardiogram (L2). Components of electrograms not identified in Fig. 1 are labeled as follows: P = action potential from Purkinje fibers at Purkinje-papillary junction; M = action potential from underlying papillary muscle. Paper speed 200 mm/sec., time lines = 40 msec. Filter settings for RA, 12/200; BH, 40/200; RPPJ, 4/200; LV, 12/200; L2, 0.1/200.

corded during the initial deflection of the electrocardiogram(5). Measurement of the time required for transmission of activity through the A-V node and for conduction in the His bundle and Purkinje system is possible. Correlation of electrograms of the specialized fibers with the standard electrocardiogram is expected to add to the value of the latter in studying disturbances of rhythm and conduction in the intact heart.

Summary. By utilizing cardiopulmonary bypass, permanent electrodes with external leads have been sutured over the bundle of His and the Purkinje-papillary junction of the right anterior papillary muscle. For the first time this technic allows the activity of the specialized conducting system to be studied directly over extended periods of time and permits observations to be made not feasible in acute experiments.

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Localization of Antigens by Autoradiography.* (26248)

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The object of this investigation was to determine the localization of injected radioactive antigens by means of autoradiography. Deposition of isotopically labeled proteins in various tissues and subcellular fractions had been reported from this laboratory (1-5) and from other laboratories (6-8). The methods used in these previous studies involved tissue homogenization and subsequent fractional centrifugation. With such manipulations, it was difficult to exclude transfer of labeled protein antigens from one to another intracellular fraction. Ambiguities of this type are avoided by using colored azoproteins which can be detected histologically in the tissue sections (9-11), or by investigating the tissues of animals injected with isotopically labeled antigens using autoradiographic technics (7,8,12). It appears that, hitherto, no attempts have been made to evaluate the autoradiographic findings on intracellular localization of injected antigens by comparison with the distribution and level of radioactive antigen in the organs of the same animals. Such experiments are described in this paper.

Materials and methods. Preparation and injection of antigens. Heavily or lightly labeled S^{35} -sulfanilazo-BGG was prepared by coupling 1.0 g BGG (Pentex Inc., Kankakee, Ill.) with 0.1 or 0.01 g respectively, of diazotized S^{35} -sulfanilic acid (2,3,7). Internally labeled S^{35} -rabbit serum albumin (S^{35} -RbSA) was obtained by injecting a rabbit with the hydrolysate of S^{35} -yeast protein and iso-

lating the albumin fraction of the blood serum as described earlier (13). A single dose of 10 to 13 mg of the heavily labeled azoproteins or of the internally labeled rabbit serum albumin was injected intravenously into mice. The lightly labeled azoproteins were injected subcutaneously into the foot pads of rabbits. The animals were killed by exsanguination from the cervical vessels. The organs of some of the injected rabbits and mice were homogenized and protein powders were prepared (13) for measurement of tissue radioactivity. In some of the experiments, liver and spleen homogenates were fractionated by differential centrifugation (1,13).

Histological procedure. Tissues were removed from the animals immediately after death, fixed in neutralized 10% formalin solution for 12 to 24 hours, washed with water, dehydrated, cleared with methylsalicylate and embedded in Fisher Tissuemat M. P. 56-58.5°. Sections of 3 to 7 μ in thickness were mounted on slides coated with gelatin-potassium alum solution and then decerated. Some sections were stained with Harris hematoxylin or Feulgen-fast green prior to autoradiography. These slides were then dipped for 20 seconds into 1% celloidin solution, allowing all excess celloidin to drain off. After drying, the celloidin layer was hardened by treatment with 80% ethanol for 1 minute, coated with gelatin and dried. Some sections were stained with Giemsa following development of the autoradiographs. Kodak Autoradiographic Permeable Base Safety Stripping Film was used in some of the experiments; in other studies, Kodak Nuclear Track Type NTB3 emulsion, obtained through the courtesy of Eastman Kodak Co., Rochester, N. Y., was employed. The slides and the liquid emulsion were warmed up to 37° in the dark room. Two or 3 drops of the emulsion were spread over the tissue section with a fine brush and the slide placed for about $\frac{1}{2}$ minute on the slide warmer at 37°. After

* Support of this work by grants of U. S. Public Health Service and Nat. Science Foundation to one of us (F.H.) and by contracts of Indiana Univ. with U. S. Atomic Energy Comm. and Office of Naval Research is gratefully acknowledged. We are also greatly indebted to Dr. R. R. Ruth for advice and cooperation. The following abbreviations are used: BGG = bovine γ -globulin, RbSA = rabbit serum albumin.

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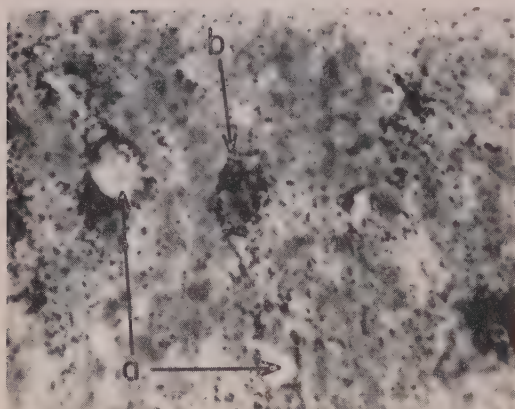


FIG. 1. Liver section of Mouse 13 which was inj. i.v. with 12.5 mg (225×10^6 cpm) of S^{35} -azo-BGG and killed after 6.5 hr. Section was stained with Giemsa and covered with stripping film. Radioactive material (a) in sinusoids and (b) in Kupfer cells. Nucleus of the Kupfer cell is free of silver grains. Exposed 21 days. $\times 700$.

drying at room temperature, slides were kept in a light-proof desiccator over calcium chloride at 5°C . Exposure times with the wet emulsion were approximately one-fifth of those required for stripping film which varied from a few days to several months.

Results. *Localization of heavily substituted S^{35} -sulfanilazo bovine γ -globulin.* Mice No. 13, 15 and 16 were killed 6.5 hours, 5 and 10 days, respectively, after intravenous injection of the antigen. Tissue sections were prepared from liver, spleen and femoral bone marrow and studied with autoradiography. Examination of the liver sections revealed silver granules concentrated (a) in the cytoplasm of Kupffer cells, forming a zone around the nucleus of each cell, and (b) in the endothelium of the sinusoids (Fig. 1). Much less radioactivity was found in the hepatic parenchymal cells. These cells also displayed more silver granules over the cytoplasm than over the nuclei. In autoradiographs of the spleen of injected animals, most of the activity was found in the red pulp, confirming earlier reports by Ingraham(7). Autoradiographs of the bone marrow sections showed the greatest number of grains to be around the islets of adipose tissue. Unequivocal identification of some cell types which contained radioactive material was impossible. However, very little activity was present in the lymphocytes.

Table I shows that the protein-bound radioactivity was concentrated in the liver and spleen, later also in kidney, whereas its concentration was low in lung, muscle and in blood. Differential centrifugation of the liver homogenate revealed that the mitochondrial protein had the highest specific activity (Table I). Similar results were obtained earlier after injection of rabbits with heavily labeled azoproteins(2).

Localization of lightly substituted, externally labeled antigens. The lightly labeled S^{35} -sulfanilazo-BGG was purified by dialysis against distilled water and precipitated by alum(14). After resuspension in isotonic saline solution, 0.3 to 0.4 ml of the suspension containing 13 to 16 mg of the antigen were injected into each of the 2 hind foot pads of 3 rabbits, No. 300, 308 and 310. The protein-bound radioactive material was present in all tissues examined (Table II). Its distribution was not very different from that found after intravenous injection(3). Elimination of the injected antigen from the blood was determined in Rabbit No. 310 by taking small samples of blood from the ear vein each day. The proteins were precipitated by trichloroacetic acid and their radio-

TABLE I. Distribution of Protein-Bound Radioactivity† after Intravenous Injection of Sulfanilazo-Bovine γ -Globulin into Mouse No. 15 and 16.

	Mouse No. 15		Mouse No. 16	
	(cpm/mg) $\times 10^{-3}$	RS _{PA} *	(cpm/mg) $\times 10^{-3}$	RS _{PA} *
Organs:				
Liver	39.7	2.4	7.1	.55
Spleen	18.2	1.1	3.7	.29
Lung	7.0	.42	.73	.057
Heart	3.0	.18	.53	.042
Blood	4.4	.26	.69	.054
Kidney	10.6	.64	5.8	.45
Liver fractions:				
Nuclear	28.1	—	8.0	—
Mitochondrial	73.3	—	15.1	—
Microsomal	26.3	—	4.7	—
Supernatant	29.0	—	5.2	—

* RS_{PA} = Relative Specific activity =
Counts/min. in 100 mg organ protein

Counts/min. inj./g animal

Weights of mouse 15 and 16 were 38 and 25 g, inj. counts/min. 63 and 32×10^6 , respectively.

† Methods used for determination of protein-bound radioactivity in organs and liver fractions have been described(1).

TABLE II. Distribution in Rabbits of Bovine γ -Globulin Labeled by Traces of Diazotized S^{35} -Sulfanilic Acid after Injection into Foot Pads.

Rabbit No.	300	308	310
Day killed	2	4	6
Wt (g)	1820	2770	2940
Inj. protein (mg)	26	28	32
Inj. cpm $\times 10^{-6}$	25.5	30.6	23.8
Urine†	56%	83%	90%

Protein-bound radioactivity:	cpm/mg	RSpA*	cpm/mg	RSpA*	cpm/mg	RSpA*
Liver	20.0	1.43	7.6	.83	1.45	.17
Spleen	23.1	1.66	8.2	.89	3.88	.44
Lung	185.0	13.2	34.7	3.8	1.02	.12
Heart	38.1	2.7	12.2	1.3	.34	.039
Bone marrow	112.0	8.0	14.1	1.5	4.4	.50
Kidneys	35.2	2.5	74.0	7.8	1.7	.20
Blood serum	1010	72	60.3	6.6	3.8	.44
Muscle	—	—	1.2	.12	.22	.025

* RSpA = Relative specific activity (see Table I).

† Total urinary excretion from time of inj. to time of death, in % of total inj. activity.

activity measured as described earlier(13). The specific activity of the blood proteins, 2, 3, 4, 5 and 6 days after injection was 133, 98, 75, 10.6 and 4.7 cpm/mg, respectively. The thymus had a specific activity of 1.03 cpm/mg. Less than one per cent of the urinary activity (Table II) was precipitable by trichloroacetic acid. Blood sera of Rabbits 300 and 308, killed 2 and 4 days after injection of the antigen, were free of precipitins, whereas the serum of Rabbit No. 310, killed 6 days after injection, showed a weak but distinct precipitin reaction when mixed with the 10-fold volume of a 0.03% or 0.01% antigen solution. Precipitin formation in this animal is also demonstrated by the steep drop

in circulating radioactive protein from 75 cpm/mg on the 4th day to 10.6 cpm/mg on fifth day after injection. Fig. 2 shows that most of the radioactive material in Rabbit No. 300 is concentrated in eosinophilic macrophages of the regional lymph node, whereas the lymphocytes contain little of the radioactivity. No increase in plasma cells was observed in sections of the lymph nodes.

Localization of internally labeled RbSA (S^{35}). Rabbit serum albumin containing S^{35} -amino acids(13) was intravenously injected into mice, and distribution of the internally labeled antigen in the spleen was examined. Autoradiographs of mouse spleen sections showed that some of the radioactivity was ex-

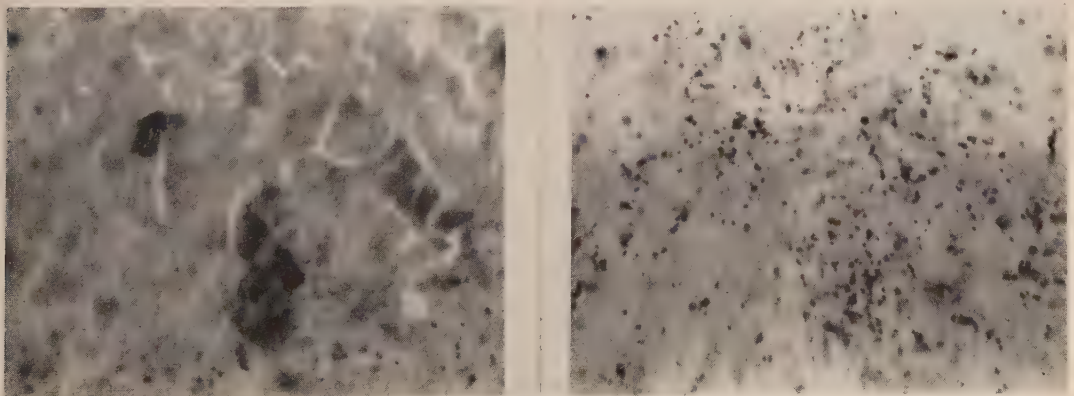


FIG. 2. Popliteal lymph node section of Rabbit 300 which was killed 2 days after inj. of BGG- S^{35} into hind foot pad. Section was stained with hematoxylin-eosin and coated with photographic emulsion. Photomicrographs with focus on cells (left) and on emulsion (right). Most of activity is concentrated in cytoplasm around nuclei of eosinophilic macrophages. Twenty-eight day exposure. $\times 700$.

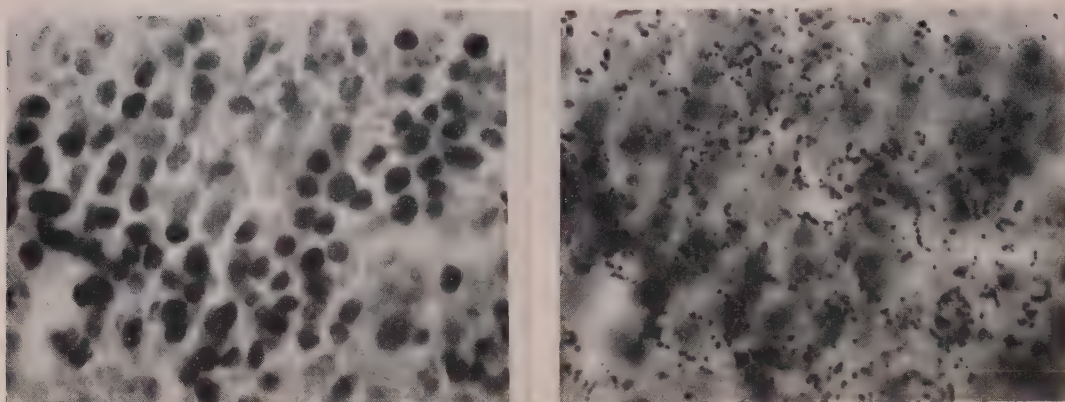


FIG. 3. Spleen section of Mouse 20 which was inj. i.v. with RbSA (S^{35}) and killed after 3 days. Section was stained with Feulgen-Fast green and coated with wet emulsion. Photomicrographs with focus on cells (left) and on photographic emulsion (right). Radioactive material is intracytoplasmic, with no intranuclear activity evident. Seventy-four day exposure. $\times 700$.

tracellular, while tracks of silver granules were found over the cytoplasm of lymphoid cells (Fig. 3). All cell nuclei remained essentially free of radioactive material.

Discussion. Using externally and internally labeled proteins, we had demonstrated earlier that both the external as well as the internal label persist as protein-bound material in tissues of rabbits for many months following intravenous injections of the protein antigens(2,5,13). This was particularly true for tissues of the reticuloendothelial system. However, the method employed in these previous studies did not indicate the cell types in which the antigens were concentrated nor sites of intracellular localization of the antigens. In the present paper, an attempt has been made to answer these questions using autoradiographic studies of animal tissues removed a few hours or days after injection of the labeled protein antigens.

We found that intravenously injected antigens were concentrated in the Kupffer cells of the mouse liver and in the red pulp of the mouse spleen. Antigens injected into the foot pads of rabbits were localized in the eosinophilic macrophages of the popliteal lymph nodes. In all these experiments, the bulk of the radioactive material was found in the cytoplasm or adsorbed to the cell membranes, whereas nuclei remained almost free of radioactivity (Fig. 1, 2 and 3). These findings agree with analogous results obtained in experiments using colored azopro-

teins(9-11) and with our earlier findings in fractions of homogenized tissues. The antigens used in all our experiments were water-soluble proteins. Previous investigations of liver and spleen homogenates demonstrated, however, that most of the radioactivity resided in the cytoplasmic granules of the liver and spleen, and not in the supernatant fraction which contains the soluble substances (1,2,7). From these observations and from the results of recovery experiments(15), we concluded that the antigen is converted to some insoluble material by combination with antibodies or by nonspecific combination with other cellular constituents such as lipids, nucleic acids or mucopolysaccharides. McMaster and Kruse(16) have drawn similar conclusions, since they were not able to detect reactive antigen in organs of injected animals with direct methods, although indirect methods clearly indicated the presence of antigen. Garvey and Campbell(17) assumed that the low serological activity of tissue-bound antigens was due to degradation of the antigen and presence of antigen fragments which were bound more or less firmly to RNA or other constituents of the cells. Our results are reconcilable with either of these views.

Summary. Analyses of the distribution of isotopically labeled antigens in the animal were combined with autoradiography of the tissues. After intravenous injection of heavily substituted S^{35} -sulfanilazo-bovine γ -globulin into mice, we found protein-bound

S^{35} in the cytoplasm of the Kupffer cells of the liver. Similarly, activity of injected bio-synthetically labeled S^{35} -RbSA was concentrated in the cytoplasm of lymphoid cells of the spleen, particularly in the red pulp. After injection of lightly labeled S^{35} -sulfanilazo-bovine γ -globulin into the foot pads of rabbits, the radioactive material was concentrated in the cytoplasm of the eosinophilic macrophages of the popliteal lymph nodes. In all these cell types, the protein-bound S^{35} was either present within the cytoplasm or adsorbed to the cellular membrane. Little intranuclear radioactivity was evident.

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Necrotizing Effects of *Staphylococcus aureus* Extract on Mouse Sarcoma 180.* (26249)

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In susceptible hosts endotoxins of gram-negative bacteria cause a variety of biological effects, such as fever, local and systemic Schwartzman reactions, necrosis of experimental tumors, as well as alterations of non-specific resistance, antibody formation, and dermal reactivity to epinephrine(1-5). The lipopolysaccharide moiety of the bacterial endotoxins seems to be responsible for the majority or all of these biological effects. In contrast to the extensive data reported on endotoxins from gram-negative bacteria, only limited information is available on similar biological effects by products from gram-positive microorganisms(3,6,7).

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Recently it was shown that a trichloroacetic acid extract from *Staphylococcus aureus* strain D alters dermal reactivity of rabbits to epinephrine(8). This effect was comparable to that observed with endotoxins of gram-negative bacteria(5) and the lipid A component of *E. coli* endotoxin(9). In view of this observation, a study of the possible tumor-necrotizing effect of the same staphylococcal extract was undertaken. As reported herein, this extract causes extensive hemorrhagic necrosis of mouse sarcoma 180.

Materials and methods. Two strains (D and 5550) of coagulase positive *Staphylococcus aureus* were used for preparation of extracts, carried out as previously described (8). The extracts were prepared at Difco Laboratories, Detroit, and obtained through

the courtesy of Dr. C. W. Christensen.

Unless otherwise specified, mouse sarcoma 180 tumors (S-180) were implanted in HaICR Swiss mice weighing between 17 and 24 g and measured according to procedures already reported(10). Single intraperitoneal injections of the bacterial extracts were given on 7th day after tumor implantation. The necrotizing effects were observed 24 hours later, and usually were evaluated on tumors exposed as shown in Fig. 1; the effect was considered either positive or negative. Results of histological examinations, performed only in a few experiments, were consistent with the visual evaluation.

Results. Intraperitoneal injection of 10

TABLE I. Necrotizing Effects of Staphylococcal Extract D on S-180.

7th day after tumor implantation			8th day after tumor implantation
Avg body wt, g	Avg tumor diam., mm	Extract, μ g/mouse	No. with hemorrhagic necrosis/Total
18.8	10.0	0*	3/20
19.3	9.8	5	9/20
18.3	11.0	10	11/20
17.3	9.4	20	13/20
18.9	11.0	40	16/20

* Control animals were given equivalent amounts of saline solution.

μ g of extract from *Staphylococcus aureus* strain D into HaICR Swiss mice bearing a 7 day-old S-180 caused marked hemorrhagic necrosis of the tumor and of the overlying skin (Fig. 1).

Histological examination of the treated tumors revealed extensive, almost total necrosis, with marked edema. When the tumor was growing very close to the skin, necrosis and pycnosis of nuclei in the epidermis were present (Fig. 2). In addition, pycnosis was seen in the Malpighian follicles of the spleen and small foci of necrosis in the regional lymph nodes corresponding to the tumor.

The effects of various amounts of the staphylococcal extract, given to HaICR Swiss mice bearing a 7 day-old S-180, were determined. Incidence of necrosis is related to the amount of extract injected (Table I). Injection of as little as 5 μ g per mouse was sufficient to cause an effect. In parallel experi-

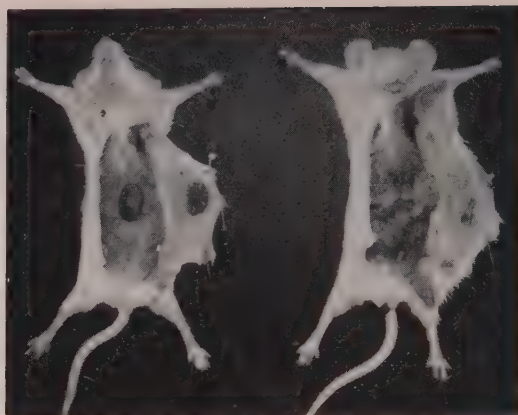


FIG. 1. Effects of extract from *Staphylococcus aureus* D on solid S-180. Left: mouse treated with 10 μ g of extract. Right: mouse treated with saline.

ments it was found that the extract was as effective as purified lipopolysaccharide from *E. coli*.

In additional experiments, the bacterial extract was injected at various times after tumor implantation (Table II). This treatment had no effect on the tumor when given on the 1st day after implantation and only a slight effect when given on the 4th day.

Experiments with rabbits have revealed that the adrenolytic drug dibenzylamine prevents development of the reaction to epinephrine elicited by the staphylococcal extract (8). For this reason, the possible influence of dibenzylamine and of epinephrine on the effects of the bacterial extract on S-180 was tested in 2 experiments. Also, the observation of the antagonistic effect of cortisone on several phenomena induced by endotoxins from gram-negative bacteria(1) prompted

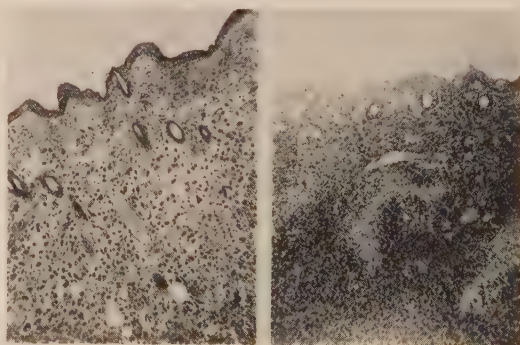


FIG. 2. Histological section of S-180 and overlying skin of mouse treated with 10 μ g of staphylococcal extract (left) or with saline (right), 100 $\times$.

TABLE II. Necrotizing Effects of Staphylococcal Extract D Injected at Various Times after S-180 Implantation.

7th day after tumor implantation		Treatment		No. with hemorrhagic necrosis/Total
Avg body wt, g	Avg tumor diam., mm	Days after implantation	Extract, μ g/mouse	
19.8	11.3	7	0*	0/26
19.8	11.9	1	20	0/20
20.3	11.8	1	40	0/30
20.6	12.0	4	20	0/20
19.7	12.2	4	40	7/30
20.0	11.2	7	20	16/20
19.5	11.8	7	40	22/30

* Control animals were given equivalent amounts of saline solution.

the study of the possible influence of this hormone on the tumor necrotizing effect of the *S. aureus* extract. The results of these experiments are shown in Table III. Epinephrine or dibenzylamine, at the doses tested, did not modify the tumor necrosis induced by the staphylococcal extract. A reduction in incidence of necrosis was observed, however, when cortisone was given daily for the 7 days between tumor implantation and injection of the extract. This antagonistic effect was not evident when the hormone was administered prior to tumor implantation or starting only 3 days before administration of the bacterial extract. Benadryl was tested in 2 experiments, in view of the possibility that this

anti-histamine drug might antagonize histamine-mediated effects of endotoxins(11). Treatment of mice with 10 to 30 mg/kg of Benadryl, 30 minutes prior to administration of the bacterial extract, did not significantly reduce incidence of tumor necrosis. In 2 additional experiments total body irradiation of the host mice (350 r/mouse, in air), given on the day before tumor implantation, did not reduce incidence of necrosis induced by the extract.

S-180, although carried routinely in Swiss albino mice, can grow also in C57Bl/6, DBA2, AKR and C3H inbred mice, even though it regresses completely after initial growth in a large percentage of animals of the latter 2 strains. It was of interest to determine whether the necrotizing effect of the *S. aureus* extract on S-180 could be seen in these inbred strains of mice, regardless of their capacity to reject the tumor. The extract was effective in all strains (Table IV).

In 2 experiments, 20 or 40 μ g of the extract had no effect on S-180 ascites in Swiss mice. Neither were hemorrhages noted in the peritoneal cavity when the tumor had grown in ascites form, nor was hemorrhagic necrosis seen in the tumor grown in solid form following subcutaneous inoculation of 10×10^6 cells per mouse.

The extract from *Staphylococcus* strain 5550, which failed to elicit the epinephrine

TABLE III. Alterations of Necrotizing Effects of Staphylococcal Extract D by Various Drugs.

7th day after tumor implantation		Treatments		8th day after tumor implantation
Avg body wt, g	Avg tumor diam., mm	Extract, μ g/mouse	Compound* and dose, mg/kg	No. with hemorrhagic necrosis/Total
23.0	8.7	0	None	0/20
22.4	8.8	20	"	13/20
23.4	8.7	0	Dibenzylamine	0/20
22.4	8.8	0	Epinephrine	0/20
22.1	8.8	20	Dibenzylamine	14/20
21.0	8.5	20	Epinephrine	11/19
23.3	14.8	0	None	0/10
22.2	12.9	20	"	10/10
21.0	15.7	0	Cortisone (I, II, or III)	0/10
21.6	12.9	20	Cortisone I	9/10
20.6	12.7	20	" II	3/10
22.4	13.4	20	" III	7/10

* Dibenzylamine and epinephrine were given intraper. on 7th day after tumor implantation. Cortisone was given subcut. daily for 7 days starting respectively 7 days prior to implantation (I), on day following implantation (II) or on 5th day following implantation (III).

TABLE IV. Necrotizing Effects of Staphylococcal Extract D on S-180 Grown in Various Strains of Mice.

Strain of mice	7th day after tumor implantation			8th day after tumor implantation
	Avg body wt, g	Avg tumor diam., mm	Extract, μ g/mouse	No. with hemorrhagic necrosis /Total
Swiss	24.0	13.4	0*	0/20
"	21.0	12.8	20	12/20
"	22.5	12.2	40	17/20
C57Bl/6	25.8	11.4	0*	0/9
"	24.7	11.1	20	4/10
"	24.9	11.9	40	8/10
DBA ₂	15.4	11.6	0*	0/5
"	16.2	11.2	20	6/9
"	19.8	11.8	40	8/10
AKR	20.8	10.5	0*	1/10
"	20.9	7.5	20	5/10
"	19.7	8.8	40	9/10
C ₃ H	18.7	12.0	0*	0/10
"	21.5	11.7	20	7/10
"	20.6	14.0	40	6/10

* Control animals were given equivalent amounts of saline solution.

reaction in rabbits(8), also had no tumor necrotizing effect on S-180 solid in Swiss mice in amounts up to 40 μ g per mouse.

Discussion. It has long been known that endotoxins of gram-negative bacteria produce hemorrhagic necrosis in certain experimental tumors. This reaction is elicited without preparatory injection of endotoxin into the tumor; in contrast, 2 injections of endotoxin are needed for production of the local Shwartzman reaction. The present study has revealed that intraperitoneal administration of a trichloroacetic acid extract from a coagulase positive strain of *S. aureus* into Swiss mice bearing 7 day-old S-180 causes marked hemorrhagic necrosis of the tumor and of the overlying skin. That gram-positive bacteria may contain a component with activities similar to those of endotoxins of gram-negative microorganisms was not entirely unexpected, since endotoxin was obtained also from hemolytic streptococci by Stetson(6). Nonetheless, the possibility has to be considered as to whether the staphylococcal extract is contaminated with endotoxin from gram-negative bacteria. The following observations strongly suggest that this is not the case: (a) the staphylococcal extract had ac-

tivity of the order of that of endotoxin from gram-negative bacteria, both in eliciting hemorrhagic necrosis in S-180 and in altering the reactivity to epinephrine of rabbits(8); (b) Jensen's staphylococcal antigen A(12), prepared from a different strain in another laboratory, was found to alter reactivity of the rabbit to epinephrine (Jensen and Neter, unpublished data); (c) altered reactivity to epinephrine was produced in rabbits also by injection of viable staphylococci under experimental conditions which preclude contamination with gram-negative bacteria or their endotoxins(8).

In the present investigation the staphylococcal extract did not cause hemorrhagic necrosis in mice bearing 1 or 4 day-old tumors, in contrast to its effect in animals bearing 7 day-old neoplasms. The fact that vascularization of S-180 is adequate only by the 4th or 5th day after implantation(13) suggests that vascularization is necessary for the extract to be effective. This suggestion is supported also by the observation that endotoxin from gram-negative bacteria is not active on tumor tissues *in vitro*(14). The fact that the *S. aureus* extract was not active on S-180 ascites is not sufficient *per se* to support the above mentioned view, because the extract was not effective even when the ascites tumor was grown subcutaneously in solid form. Strain-specific, host defense mechanisms do not appear to be involved in the necrosis of S-180 caused by the extract, since necrosis occurred also in inbred strains of mice, regardless of their capacity to reject S-180 implants.

Small scattered areas of necrosis are noted microscopically in untreated 7 day-old S-180, which increase progressively and become massive 2 to 4 weeks after implantation. The question arises, therefore, whether the marked effects of the bacterial extract in 1 week-old S-180 is related to the necrotic processes which occur in older tumors. The possibility may be considered that a physiological mediator may be operative in either spontaneous or extract-induced necrosis of S-180. Data reported here indicate that epinephrine is probably not an essential mediator in the necrosis caused by the extract.

In fact, this effect was neither enhanced by epinephrine nor antagonized by dibenzylamine, a potent adrenergic blocking agent, when these drugs were used in doses effective in rabbits(8). Similarly, the observation that Benadryl, an antihistaminic agent, did not significantly antagonize the effects of the extract on S-180 suggests that histamine does not play a critical role in the pathogenesis of the tumor necrosis studied. The antagonistic effect of cortisone, although difficult to explain at present, is consistent with results obtained by others in different systems and suggests possible interference with the effects of endotoxins by this hormone(1).

The observations described herein on the effects of an extract from *Staphylococcus aureus* on S-180 provides additional evidence that gram-positive bacteria may contain a substance with biological activities similar to those of endotoxins of gram-negative microorganisms.

Summary. 1) A trichloroacetic acid extract of *Staphylococcus aureus*, strain D (5 to 40 µg/mouse), in 24 hours caused extensive hemorrhagic necrosis of Sarcoma 180 in HaICR Swiss mice when injected intraperitoneally 7 days after tumor implantation. The extract was ineffective against tumors 1 to 4 days after implantation. 2) The effect was also produced in Sarcoma 180 grown in C57Bl/6, C3H, AKR and DBA₂ mice. 3)

Epinephrine, dibenzylamine and the antihistaminic drug Benadryl, in the doses used, did not alter the necrotizing activity of the extract. Cortisone, however, reduced the incidence of the effect.

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Role of the Sex Hormones in Peptic Ulcer.* (26250)

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Recent clinical observations have stimulated interest in the relationship of abnormalities of the various endocrine organs and occurrence of acid-peptic ulceration. It has been well established clinically that duodenal ulcer occurs much more frequently in males

than in females. A recent report of peptic ulcer occurring in 2 brothers with primary testicular atrophy(1) is of unusual interest. Peptic ulceration is observed frequently in patients with hepatic cirrhosis(2). During pregnancy progressive decrease in gastric secretory volume is followed by a lower incidence of peptic ulcer(3). Pregnancy and cirrhosis are associated with an increase of circulating estrogens.

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TABLE I.

Group	Rx	No. of dogs	Sex	No. showing ulceration at time of death or sacrifice	Duration of treatment until death or ulceration (days)	% of group ulcerating
I	H	12	7 ♂ 5 ♀	6 dogs had duodenal ulcer(s) of which 1 had perforated (5 male, 1 female)	20.3	50.0
II	H T	12	7 ♂ 5 ♀	9 dogs had duodenal ulcer(s) of which 1 had perforated; one had a gastric ulcer (6 male, 4 female)	12.6	83
III	H Stil	12	6 ♂ 6 ♀	10 dogs had duodenal ulcers (4 male, 6 female)	9.3	83

H—histamine, T—testosterone, Stil—stilbestrol.

These clinical observations do not provide a satisfactory indication of what role, if any, the sex hormones play in originating or perpetuating acid-peptic ulceration. The greater frequency of duodenal ulcers in male human subjects, the equal incidence of ulcer in both sexes prior to puberty(4) and recent reports of endocrine abnormalities concomitant with fulminating peptic ulcer(5) have prompted the following experiments. In this study, the effects of testosterone and diethyl stilbestrol upon histamine induced ulceration and gastric secretion are evaluated. The study is divided into two sections. The first concerns the effect of testosterone and diethyl stilbestrol on histamine induced ulceration.

Methods and materials. Adult mongrel dogs of both sexes were divided into 3 groups. All dogs received daily intramuscular injections of 30 mg of histamine in beeswax until death or termination of experiment(6). Group I (12 dogs, 6 males and 6 females) received no additional medication. Group II (12 dogs, 7 males and 5 females) received a daily injection of 100 mg of testosterone cyclopentyl-propionate in oil in addition to the histamine. Group III (12 dogs, 6 males and 6 females) were given 5 mg of diethyl stilbestrol daily in addition to histamine.

All dogs were maintained in individual cages and received a standard kennel diet and water *ad libitum*. Injections were made daily at the same time in the afternoon. The 4 dogs that did not die while receiving 30 mg of histamine in beeswax alone were sacrificed, one after 45 days, the other 3 after 90 days.

In all dogs, the stomach and duodenum were examined grossly for evidence of peptic ulcers; when doubt as to their presence existed, microscopic sections were made of any suspicious area.

Results. In Group I (histamine), 6 of the 12 dogs (5 males, 1 female) showed duodenal ulcers at time of death (Table I). The interval required to cause death from ulcers or to produce peptic ulceration in dogs averaged 20.3 days. One dog died after 28 days and another after 29 days without developing ulcers and the remaining 4 had no ulcers upon sacrifice, at 45 or 90 days. This incidence of ulceration is somewhat lower than that previously reported and may be the result of allowing the dogs to eat following histamine injection.

Of the 12 dogs in Group II (histamine and testosterone), 10 had ulcers at time of death (6 males and 4 females), the occurrence of which averaged only 12.6 days from onset of treatment (Table I). The other 2 dogs died prior to 45 days without ulceration (one at 3 days and the other at 36 days.) In Group III (histamine and stilbestrol), 10 of the 12 dogs (4 males, 6 females), died with well developed duodenal ulceration in an average of 9.3 days (Table I). Of the remaining 2 dogs, one died in 3 days and the other in 10 days without developing an ulcer.

The second portion of the study concerns the effect of stilbestrol and testosterone on gastric secretion.

Methods and materials. Five adult female and 4 adult male mongrel dogs weighing 8-12

TABLE II. Stilbestrol. Change in Heidenhain Pouch Secretion.

Dog	Sex	Volume, %	Free acid, %
366	♀	+18	-14
124	"	+13	0
582	♂	-11	-11
589	♀	-27	-41
827	"	-12	0
616	♂	-50	-51
	Avg	-10	-19.5

kg were used. Twenty-four hour secretions from the Heidenhain pouch in each animal were collected for at least 20 days prior to administration of stilbestrol or testosterone. All animals were given a diet of 300 g of lean cooked meat and 100 g of dried kennel rations daily. Water was given *ad libitum*. Following initiation of injections, 24 hour secretions were again collected for a period of 20 days. An interval of 10 days was allowed when the hormone administered was changed. All specimens were analyzed for volume, pH, free acid, and pepsin.

Six dogs (2 males and 4 females) received 5 mg of diethyl stilbestrol daily and 5 dogs (2 males and 3 females) received 100 mg of testosterone cyclopentylpropionate in oil daily. Two dogs received diethyl stilbestrol for 20 days and after a 10-day rest period, received 100 mg of testosterone cyclopentylpropionate for an additional 20 days. All injections during this portion of the study were given at 7:30 a. m. daily.

Results. The dogs receiving stilbestrol showed very little change in amount and character of pouch secretion (Table II). The volume dropped an average of 10% and average free acid decreased by 19.5%. No change occurred in pepsin secretion. Dogs in the testosterone series showed similar results (Table III). Average volume decreased

11.6%, and free acid 6.4%. Again, there was no difference in pepsin secretion.

Grouping the dogs by sex, the 4 female dogs that received stilbestrol showed no change in volume of secretion and average decrease of free acid was 14%. The 3 female dogs that received testosterone showed an average decrease in volume of 10%, and average increase of free acid of 13%. The 2 male dogs receiving testosterone showed an average decrease in volume of 19% and a decrease in free acid of 45%. These changes in secretion obviously are small and do not appear to be significant.

Discussion. Both testosterone and stilbestrol show obvious abatement of peptic ulcer provocation by histamine. It has been shown previously that other drugs such as pitressin (7), nitroglycerin (8), and caffeine (9) can potentiate the effect of histamine on the duodenal mucosa. The effectiveness of the aforementioned drugs has generally been attributed to changes induced on the local vascular system producing mucosal ischemia and therefore enhanced susceptibility to the gastric juice. Since the effect of testosterone and stilbestrol on gastric pouch secretion is not significant, hypersecretion is probably not the important factor in the observed abatement of the histamine provoked ulcer noted in these experiments. The underlying mechanism for potentiation of the histamine ulcer by these 2 hormones remains obscure. Kowalewski *et al.* (10) have quantitated the production of mucopolysaccharides in the stomach of rats by a radioactive sulfur technic and have shown a decrease in production of these substances following administration of testosterone.

Summary. Histamine induced ulceration in the dog is enhanced by daily administration of 5 mg of diethyl stilbestrol or 100 mg of testosterone cyclopentylpropionate. Secretion from Heidenhain pouches in dogs is not significantly changed by daily administration of these drugs.

TABLE III. Testosterone. Change in Heidenhain Pouch Secretion.

Dog	Sex	Volume, %	Free acid, %
366	♀	-9	+27
124	"	+13	+38
1811	"	-24	-7
1503	♂	-49	-42
1183	"	+11	-48
	Avg	-11.6	-6.4

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Induction of Mammary Secretion in Rats by Electrical Stimulation.* (26251)

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This laboratory has recently demonstrated that many agents can induce mammary growth and/or secretion in estrogen-primed rats, including adrenergic and cholinergic drugs(1,2), tranquilizers(3,4), morphine(5), carcinogens(2), rat hypothalamic tissue(6), electrical stimulation of the uterine cervix (5) and several nonspecific stresses(7). Most of these agents are believed to act through the CNS but others may directly stimulate the anterior pituitary to release prolactin and ACTH. Most of these agents have produced adrenal cortical stimulation as indicated by reduced thymus weight, and both prolactin and ACTH have been shown to be essential for initiating mammary secretion in intact, estrogen-primed rats(7). It was of interest to see whether direct electrical stimulation of the head, nasal mucosa, lumbar region and nipples could also release sufficient prolactin and ACTH to induce mammary secretion in estrogen-primed rats.

Methods. Virgin female rats (Carworth) weighing between 200-300 g were injected subcutaneously with 10 μ g estradiol per day for 10 days to produce mammary lobule-alveolar development(4). For the next 5 days, 4

groups of 5 rats each were electrically stimulated for 30 seconds twice daily in the regions of the head, nasal mucosa, lumbar area and nipples, respectively. Ten control rats received injections of physiological saline twice daily. An electrodyne stimulator with fine needle point electrodes, set at a frequency of 20 cycles/second, was used. The electrodes used for different groups were the same. The alternating current was just detectable on the finger tips and produced only slight struggling by the rats. Electrical stimulation of the head was effected by piercing the 2 electrodes through the skin over the frontal bones; of the lumbar region by piercing the electrodes through the skin of the 2nd and 3rd lumbar vertebrae; of the nasal mucosa by placing the electrodes in the nasal openings; of the nipples by inserting one electrode in the dermis of the teat and the other in the skin surrounding the nipple. On the 16th day, all rats were killed and the right inguinal mammary glands were removed, fixed in Bouin's fluid, sectioned at 6-7 μ and stained with H and E stain. The sections were examined microscopically and assigned ratings of 0 to 3.00 in units of 0.25, depending on amount of mammary secretion observed. Adrenals, thymus, ovaries and uterus were also removed and weighed.

Results. The mammary glands of the saline-injected controls (Group 1) regressed from a lobule-alveolar system at 10 days to practically a bare duct system by the 16th day, with no evidence of secretion (Table I).

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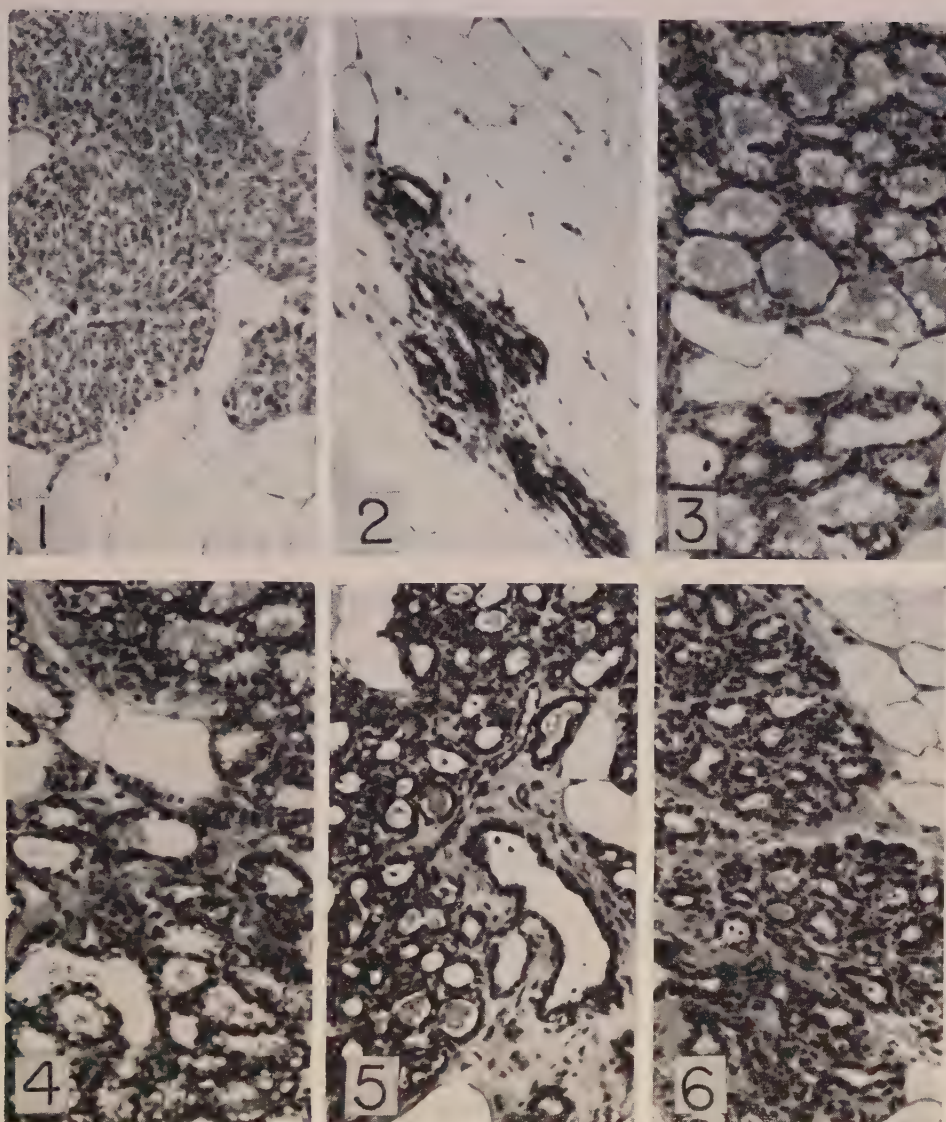


FIG. 1-6. Histological sections of mammary tissue from treated rats. (1) Estradiol 10 days. Note lobule-alveolar growth. (2) Estradiol 10 days, saline 5 days. Note mammary regression. Other rats were given estradiol 10 days, followed by electrical stimulation of (3) nasal mucosa, (4) lumbar area, (5) head, (6) nipples. $\times 125$.

All electrically-stimulated rats (Groups 2-5) showed varying degrees of mammary secretion and maintenance of lobule-alveolar structure, which were more pronounced in the rats stimulated in nasal mucosa and lumbar region than in those stimulated on head or nipples (Fig. 1-6). No inflammatory or edematous reaction was observed at site of insertion of the electrodes. Thymus glands of all electrically-stimulated groups were sig-

nificantly reduced in weight below control values, but none of the other organs showed significant weight differences.

Discussion. The present results agree with and extend previous work showing that electrical stimulation of the uterine cervix initiates mammary secretion in estrogen-primed rats(5). Stimulation of nasal mucosa and lumbar regions was more effective than stimulation of nipples or head. This

TABLE I. Effects of Electrical Stimulation on Initiation of Mammary Secretion in Estrogen-Primed Rats.

Group and No. of rats	Region stimulated for 5 days	No. with secretion	Avg secretion rating*
1 (10)	Controls, saline 2× daily	0	0
2 (5)	Head, stim. 2× daily	5	0.5
3 (5)	Nasal mucosa, stim. 2× daily	5	1.2
4 (5)	Lumbar area, stim. 2× daily	5	0.9
5 (5)	Nipples, stim. 2× daily	4	0.4

* Range = 0 to 3.00.

may be due to greater concentration of nerve fibers in the former sites. Frequent suckling of the nipples of virgin rats by fresh litters has been shown to induce mammary growth and lactation(8), and in postpartum rats to elicit release of prolactin and ACTH(9), but nipples of the estrogen-primed rats were not as well developed as in these rats. Since all electrically-stimulated groups showed increased adrenal cortical function as indicated by a significant decrease in thymus weight, and both ACTH and prolactin are needed to initiate mammary secretion in estrogen-primed rats(7), it can be concluded that both hormones were released by electrical stimulation.

It is of interest that pseudopregnancy has been induced in sexually mature rats by electrical stimulation of the skull(10) and uterine cervix(11); and in sexually mature rabbits by electrical stimulation of head and lumbar region(12). Sexual maturation in prepubertal rats has also been hastened by electrical stimulation of the uterine cervix(13). Thus in the rat, electrical stimulation of some tissues apparently induced release of FSH and LH in addition to ACTH and prolactin. It is probable that oxytocin and vasopressin are also released, but there is no evidence that either of these hormones can induce mammary secretion in rats(5,14).

The effects on the anterior pituitary are believed to be mediated through the autonomic and CNS. Thus, interruption of sympathetic pathways during electrical stimulation of the uterine cervix prevents pseudopregnancy in the rat (15), while transplantation of the pituitary to a non-cranial site(16) or

injections of rat hypothalamic tissue(6) induces mammary secretion in estrogen-primed rats.

Summary. Mature virgin female rats were injected subcutaneously with 10 µg estradiol daily for 10 days to produce mammary lobule-alveolar development. For the following 5 days, all except 10 controls were electrically stimulated twice daily for periods of 30 seconds on head, lumbar region, nasal mucosa and nipples. Control rats showed regression of the mammary glands to a bare duct system and no secretion, whereas almost all of the electrically stimulated rats showed variable degrees of mammary secretion and maintenance of lobule-alveolar structure. Adrenal cortical stimulation in all electrically-stimulated groups was indicated by significant decreases in thymus weight. It is concluded that electrical stimulation of these areas in the rat induces release of both prolactin and ACTH, probably through autonomic and CNS pathways.

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Life Span of Mouse Blood Platelets. (26252)

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Little information about mouse blood platelets is available even though the mouse is widely used for mammalian experimentation, including hematological research. In this study we undertook to determine the life span of mouse blood platelets by a radioisotopic method previously used in rats(1).

Methods. Male BDF₁ mice weighing 23-28 g were injected intraperitoneally daily throughout each experiment with 1 μ c of Na₂S³⁵O₄/g of body weight, except that at the first injection they received 135 or 115% of this dose. The purpose of daily injections was to maintain a relatively constant day-to-day pool of radioisotopic label for incorporation into platelets. Injections were made on the basis of daily body weights, except in the first experiment where initial weight was used. In some experiments carrier sulfate (added as Na₂SO₄), usually 0.6 μ g SO₄/ μ c of S³⁵, was added to the radioactive sulfate to facilitate its distribution throughout the blood. Mice were killed at intervals after first injection of sulfate and always 24 hours after last injection. In some experiments, peripheral platelet counts were taken at this time. The mice were injected intraperitoneally with 0.5 ml of a heparin-Nembital-saline solution† and were bled about 10 minutes later from the heart into siliconed syringes containing 0.05 ml of 1.0% disodium ethylenediaminetetraacetate dihydrate in 0.7% saline. The blood of each mouse was expressed into a 60 $\times$ 10 mm siliconed tube and mixed with an equal volume of 3.5% PVP,‡ a plasma expander. Platelets were then separated from the blood by differential centrifugation, first by centrifuging slowly to obtain a plasma-PVP-platelet layer, then by

decanting this into another tube and centrifuging at a more rapid rate to obtain a platelet button in the bottom of the tube. Platelets of each mouse were washed twice with 0.5 ml of saline and resuspended in approximately 0.2 ml of saline. Numerical white blood cell and platelet counts were made on each platelet suspension. One 100 μ l sample was placed on a planchet and dried for counting in a windowless gas flow counter. The radioactivity of two 100 μ l samples of the platelet-free plasma-PVP component and of one 200 μ l sample of each wash was also determined. Very few to no white blood cells were present in the platelet suspensions. Radioactivity per average platelet was determined by dividing radioactivity of a platelet sample by total number of platelets in that sample; the resulting values were multiplied by 10⁷ for use in Fig. 1. Actual radioactivity counts of samples of platelets were about

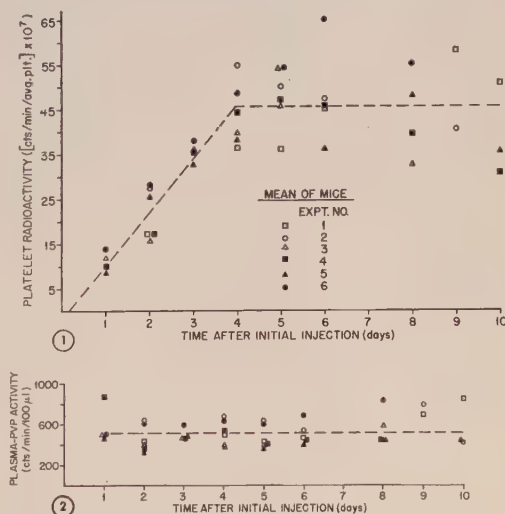


FIG. 1. Radioactivity of mouse blood platelets during a period of daily inj. of Na₂S³⁵O₄. Symbols denote mean platelet activity of mice (3 or 4) in each experiment at each interval. Total No. of mice was 136. For dashed line see text.

FIG. 2. Radioactivity of plasma-PVP during period of daily inj. of Na₂S³⁵O₄. Symbols denote means in each experiment at each interval. Dashed line represents grand mean of all mice.

* Operated by Union Carbide Corp. for U. S. Atomic Energy Comm.

† 1 cc heparin (1000 U.S.P. units); 1 cc Nembital (60 mg); 10 cc saline.

‡ PVP: Polyvinylpyrrolidone or Vinisil was kindly supplied by Abbott Laboratories.

300 to 2000 cpm (background was 25 cpm). Total number of platelets in 100 μ l samples counted for radioactivity was in the range of 1.5 to 3.5×10^8 .

Results. The radioactivity of platelets increased rapidly during the first 4 days, and was widely variable thereafter, although apparently without definite upward or downward trends. Mean platelet activity of the mice in each of 6 experiments is presented in Fig. 1. The rising dashed line was drawn by eye through the means obtained on days 1-3, and the horizontal dashed line was determined by averaging platelet activities of all 67 mice on days 5-10. Rate of increase toward the plateau value during the initial period was about 26% per day. Although variation among mice was large, mean plasma activity was quite constant throughout the 10-day period (Fig. 2). Radioactivity of 200 μ l samples of the second platelet washes was low, in the general range of once to twice background. The amount of S^{35} injected was less than that which produces detectable hematologic(2) or histologic(3) damage. In one of our experiments no damage was evident in the bone marrow of sections of femurs of mice given 8 or 10 daily injections.

Discussion. Incorporation of sulfate into platelets appears to take place primarily through their precursors, the megakaryocytes. In rats this was suggested by a gradual, rather than immediate, increase in S^{35} activity in platelets of peripheral blood over a 3-day period after a single injection of $Na_2S^{35}O_4$, and by occurrence of heaviest labeling of the megakaryocytes, as seen in autoradiograms of bone marrow smears, about 2 days prior to peak activity of peripheral platelets(4). The sulfate becomes incorporated in platelets into a sulfated mucopolysaccharide(5).

Survival time of platelets in the peripheral blood of rats was previously studied essentially by the method used here, and 3 pos-

sible interpretations of the experimental data were considered: 1) that rat blood platelets have a life span; 2) that platelets are removed from circulation by random destruction; and 3) that both kinds of removal occur(1). On the basis of degree of correspondence of the experimental points to a straight (life span) or a curved (random loss) line it was concluded that the data were most consistent with a life span concept. We believe that a similar situation exists in mice, although the greater amount of variation in the mouse data results in a somewhat greater deviation of the experimental points from the lines in Fig. 1. By our interpretation, the rate of increase of platelet radioactivity toward the plateau level, 26% per day, indicates a life span of platelets in the peripheral circulation of BDF₁ mice of approximately 4 days. The mouse platelet survival is about the same or slightly shorter than that of rats.

Summary. In male BDF₁ mice injected daily with $Na_2S^{35}O_4$, radioactivity of the circulating platelets increased at a rate of 26% per day, reaching a plateau in 4 days. From the time required to attain maximum labeling of the circulating platelet population, the life span of mouse platelets is estimated to be approximately 4 days.

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Differences in Efficiency of Energy Transfer in Mitochondrial Systems Derived from Normal and Failing Hearts.*† (26253)

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In contrast to the voluminous literature on the problem of congestive heart failure from both the clinical and pathophysiological viewpoint, there is a dearth of information on the basic mechanism of congestive heart failure from the viewpoint of bioenergetics. A method for producing congestive heart failure in animals has recently become available and has afforded the opportunity to study one important type of congestive heart failure, *i.e.*, decreased output failure, backward failure, or increased forward resistance failure, etc., with employment of biochemical technics(1,2).

Many theories which have been espoused to explain the genesis of congestive heart failure are adequately reviewed elsewhere(1,3). Starling's Law of the heart is utilized in nearly all instances except those where there is failure in energy production, *e.g.*, beriberi; energy transport, *e.g.*, thyrotoxicosis and energy utilization, *e.g.*, marked coronary artery disease with fibrosis.

The data presented herein are an attempt to demonstrate some metabolic differences in mitochondrial systems derived from normal

guinea pig hearts and hearts from guinea pigs in experimental congestive heart failure. Also, further information was sought on bioenergetics as related to Starling's Law of the heart(4).

Experiments were designed to obtain further information on these two important questions from the biochemical viewpoint. Heart mitochondrial systems were obtained from guinea pigs in which congestive heart failure was produced experimentally. The effects of adding various cofactors known to affect the metabolism of the heart mitochondrial system were noted on the QO_2^N values and P/O ratios in heart mitochondrial systems prepared from normal guinea pigs and experimental guinea pigs.

Methods and materials. Hartley Albino male guinea pigs weighing circa 400 g were divided into 2 groups. In one group (usually 6-8 animals) congestive heart failure was produced by restricting the aortic outflow *via* an intrathoracic incision(2). The remaining group of 6-8 animals served as a control. Heart mitochondrial systems were prepared by a method of Maley and Plaut(6). The mitochondria prepared showed no oxidation without substrate and there was no demonstrable ATPase activity(7).

In the oxidation and phosphorylation studies, each Warburg vessel contained the following medium (variations from this composition are indicated in the text and tables): (a) Main compartment: phosphate buffer pH 7.4 (0.1 M) 50.00 μ M, magnesium chloride (0.1 M) 10.00 μ M, ATP (0.1 M) 6.00 μ M, cytochrome C (0.003 M) 0.03 μ M, mitochondria 0.80 ml (equivalent to 0.5 mg of nitrogen), substrate α -ketoglutarate (0.2 M) 20.00 μ M, sucrose (0.25 M) to a final volume of 3.0 ml. (b) Center well: 5N potassium hydroxide 0.20 ml. (c) Side-arm: hexo-

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† The following symbols will be used throughout this paper:

CoA	—	Coenzyme A
DPN	—	Diphosphopyridine nucleotide (oxidized)
TPN	—	Triphosphopyridine nucleotide (oxidized)
TCA	—	Trichloroacetic acid
ATP	—	Adenosine triphosphate
ATPase	—	Adenosine triphosphatase
C.H.F.	—	Congestive heart failure
QO_2^N	—	μ l of oxygen consumed per hr per mg of mitochondrial nitrogen
P/O	—	$\frac{\text{Micromoles of orthophosphate consumed}}{\text{Microatoms of oxygen utilized}}$
ΔP	—	Micromoles of orthophosphate consumed by the system
ΔO	—	Microatoms of oxygen utilized by the system

‡ The abbreviations are listed separately.

kinase (400 Cori units) in 2% glucose.

Hexokinase was prepared by the Colowick and Darrow[§] method and purification accomplished to the Bentonite step. The medium components were added to flasks chilled in an ice-bath. The freshly prepared suspension of mitochondria (containing circa 0.8 mg of nitrogen per flask) was added to the main compartments just prior to attaching the flasks to the manometers. After an equilibration period of 12 minutes at 30°C, the hexokinase-glucose mixture was added to the main compartment, the zero time flask was removed, and manometric readings of the incubated samples were made, in sequence at one minute intervals. Each sample was run for 15 minutes and occasionally for 20 minutes. At termination of the experiment each flask was quickly removed from the bath, placed in ice, and the reaction was stopped immediately with 2.0 ml of ice-cold 20% trichloroacetic acid. The coagulated protein was removed by centrifugation. The orthophosphate content of the protein-free filtrate was determined by the method of Lowry and Lopez(8). All analyses and experimental flasks were done in duplicate and occasionally in triplicate to assure reliable results. Nitrogen was determined by a modification of Johnson's technic(9).

Results. Table I summarizes the results obtained by adding, either in combination or individually, various cofactors such as CoA, DPN and TPN to mitochondrial preparations from both normal and failure guinea pigs. $Q_{O_2}^N$ values and P/O ratios showed little difference between heart mitochondrial systems derived from the normal and experimental guinea pigs, *i.e.*, 1170 and 1032, and 2.90 and 2.91 respectively. Addition of the individual cofactors revealed certain differences in $Q_{O_2}^N$ value and P/O ratio. In the mitochondrial systems derived from both the normal and failure animals, there was a stimulation of respiration with addition of CoA and DPN added separately, but P/O ratio was affected only slightly. Addition of CoA

TABLE I. Effect of Adding Various Cofactors Either Singly, or in Combination, on Respiration and Efficiency of Phosphorylation by Cardiac Mitochondrial System Prepared from Normal and Failure Guinea Pigs Employing α -Ketoglutarate as Substrate.*

	Normal†		Failure‡	
	$Q_{O_2}^N$	P/O	$Q_{O_2}^N$	P/O
Complete system	1170	2.90	1032	2.91
<i>Idem</i> + CoA + DPN + TPN	1480§	2.32	1524¶	1.95**
" + CoA	1385	2.88	1256	2.73
" + DPN	1163	2.80	1032	2.95
" + TPN	380	1.25	305	1.07
" + CoA + DPN	1403	2.95	1595	2.46
" + CoA + TPN	1096	2.63	1069	2.64

* For each experiment 6-8 guinea pig hearts were employed. Figure represents avg of 3 exp.

† Values represent avg of 4 exp.

‡

§ Represents increase of 26.5% over normal values in complete system only. "t" test = 6.03; $p = >.001$.

|| Represents a decrease of 20.0% as compared with normal values in complete system only. "t" test = 3.5; $p = >.01$.

¶ Represents increase of 47.5% over normal values in complete system only. "t" test = 20.4; $p = >.0001$.

** Represents decrease of 33.0% as compared with normal values in complete system only. "t" test = 6.2; $p = >.001$.

Test of significance employed is based upon Suedecor(12).

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{(s.e._1)^2(N_1 - 1) + (s.e._2)^2(N_2 - 1)}{N_1 + N_2 - 2} \times \frac{1}{N_1} + \frac{1}{N_2}}}$$

$\bar{X}$ = mean value; N = total No. in sample; s.e. = stand. error of mean value.

and DPN together and CoA and TPN together stimulated respiration to an even greater degree but did not lower the P/O ratio appreciably. TPN alone did not stimulate the respiration and appeared to decrease the efficiency of oxidative phosphorylation in both groups. Whether this is an artifact or real cannot be answered at this juncture.

The effect of adding all cofactors, *i.e.*, CoA, DPN and TPN to both mitochondrial systems had a similar effect in both groups, *i.e.*, stimulation of respiration and reduction in efficiency of oxidative phosphorylation. These results are emphasized in Table I.

$Q_{O_2}^N$ values increase under this treatment in both the normal and failure mitochondrial

§ The author wishes to thank Dr. Colowick and Dr. Darrow for permitting the author to use the method prior to publication.

TABLE II. This Table Summarizes ΔP , ΔO , and $Q_{O_2}^N$ Values and P/O Ratio in Both Normal and Experimental Failure Animals with and without Addition of Cofactors CoA, DPN and TPN. Amount of energy derived from the mitochondrial systems is also given.

	ΔP	ΔO	P/O	$Q_{O_2}^N$	Calories, energy $\sim P^*$
Normal	27.08	9.29	2.90	1170	306×10^4
Normal + cofactors	26.98	11.70	2.32	1480	306×10^4
Failure	21.12	7.15	2.91	1032	268×10^4
Failure + cofactors	21.14	10.82	1.95	1524	265×10^4

* Calculated on basis of $Q_{O_2}^N$ value.

$Q_{O_2}^N$ value

11.2 (for conversion of molecular oxygen to atomic oxygen)

$\times 10,000$ (assuming each terminal bond of phosphorus is equivalent to 10,000 calories)

$\times$ Oxidative phosphorylation (P/O ratio)

=

Sample calculation:

$$\frac{1170}{11.2} \times 10,000 \times \frac{2.90}{1} = 306 \times 10,000 =$$

systems, but P/O ratios decrease in both instances. $Q_{O_2}^N$ values increase to a larger degree in the failure systems, 47.5% *vs.* 26.5%, while P/O ratio falls to a great degree, 33.0% *vs.* 20.0% in the same system.

In the studies with mitochondrial systems derived from normal and failure animals, the calculated energy derived does not change with addition of cofactors. This is of particular interest in the case of the experiments with mitochondrial systems derived from failure hearts where there is a considerable drop in the P/O ratio but the inordinate rise in $Q_{O_2}^N$ value in the same system compensates for the loss in efficiency of phosphorylation (Table II).

Discussion. The syndrome of congestive heart failure produced experimentally in the guinea pig is very similar to that observed in humans with chronic aortic stenosis. This experimental type of congestive heart failure differs from that produced by Alexander *et al.* (10), and Barger (11), in that in the former experiments the abdominal aorta is compressed while in the latter the tricuspid valve is avulsed and the pulmonary artery com-

pressed. In the former the failure produced shows more hypervolemia while the latter is more of chronic right heart failure.

These data pose an interesting question which may have some relationship to Starling's Law of the heart (4). One of the most interesting observations in the phenomenon of Starling's Law of the heart is that cardiac oxygen consumption continues to increase during the increased systolic ejection concomitant with cardiac efficiency and continues to increase in spite of the decreased systolic ejection and subsequent decrease in cardiac efficiency (5). Several questions arise from these observations: (a) Why does oxygen consumption continue to increase despite the decreased cardiac efficiency? (b) What is the relationship between oxygen consumption and source of energy required by the heart to continue ejecting the required blood?

A possible explanation for the increased oxygen utilization in spite of decreased cardiac efficiency as observed by Evans *et al.* (5) may be apparent from the experiments presented herein. It is possible to compare certain aspects of bioenergetics when heart mitochondrial systems are prepared from animals in congestive heart failure and compared with similar systems derived from normal animals, (a) under conditions which may closely parallel those observed *in vivo* and (b) under conditions of biochemical stress or stimulation. With the substrate α -ketoglutarate, $Q_{O_2}^N$ values and P/O ratios are virtually the same in both groups (Table I). Under the influence of the combination of various cofactors, *e.g.*, CoA, DPN and TPN, $Q_{O_2}^N$ value of the heart mitochondrial systems prepared from animals in congestive heart failure rises to 1524 while that of the heart mitochondrial system prepared from the normal group rises to 1480. The most important difference exists, however, in the P/O ratios. The P/O ratio in the experimental group decreases to 1.95 while that in the normal group decreased slightly to 2.32.

One interpretation of these data is as follows. In the normal guinea pig heart, the inordinate increase in rate of respiration, as

evidenced by the increases $Q_{O_2}^N$ value does not change the efficiency of phosphorylation. Thus, amount of work may be increased, efficiency remains constant, and total calories produced are the same (Table II). In the heart mitochondrial systems derived from the experimental animals, there is an even greater increase in respiration rate, but there is less available energy per atom of oxygen because the efficiency of phosphorylation is decreased. However, in spite of this apparent loss of efficiency, total amount of energy obtained is the same (Table II).

It thus may be possible to interpret Starling's Law in biochemical terms. The energy which the heart (whether normal or in failure) requires to perform its normal cardiac output is provided by the coupling of oxidation and phosphorylation. However, when there is an uncoupling or loss in efficiency of rate of phosphorylation from oxidative mechanisms, as is the case of mitochondria derived from failing guinea pig hearts under the influence of cofactors, greater oxidation will be required to produce the equivalent amount of energy for the usual amount of cardiac work (Table II). Thus it may be reasonable to suggest a relationship between the physiologic observation that oxygen consumption increases in the failing heart, *viz.* Starling's Law, with the observation of the increased $Q_{O_2}^N$ value of mitochondria derived from failing hearts under the influence of cofactors.

Summary and conclusions. 1. Oxidation rates employing α -ketoglutarate as substrate and efficiency of phosphorylation coupled with oxidation were studied in heart mitochondrial systems prepared from normal guinea pig hearts and hearts from guinea pigs in congestive heart failure. 2. $Q_{O_2}^N$ values were 1170 and 1032 for the normal guinea pig hearts and experimental guinea pig heart mitochondrial systems for α -ketoglutarate oxidation respectively. P/O ratios were 2.95

and 2.91 respectively. 3. Addition of cofactors (CoA, DPN and TPN), increased $Q_{O_2}^N$ values from 1170 to 1480 in the normal heart mitochondrial systems and from 1032 to 1524 in the mitochondrial systems derived from animals in experimental congestive heart failure. P/O ratios decreased slightly in the normal systems (2.95 and 2.32) while the P/O ratio decreased from 2.91 to 1.95 in the animals in congestive heart failure. 4. The increase in energy production parallels the overall increase in $\sim$ phosphorus during addition of cofactors in both normal animals and failure animals. Although the efficiency of phosphorylation in the mitochondrial system derived from failing hearts is markedly decreased during the influence of the cofactors, energy production is maintained by the inordinate increase in respiration. The possible relationship of this observation to basic physiologic mechanisms is suggested.

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Effect of Acetazolamide on Passage of Protein from Cerebrospinal Fluid to Plasma.* (26254)

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The enzyme, carbonic anhydrase has been reported in numerous parts of the central nervous system including the choroid plexus of the cat brain(1). In view of the known action of acetazolamide as a carbonic anhydrase inhibitor, one might expect acetazolamide to have an effect on CSF dynamics. Acetazolamide administration has been reported to reduce CSF formation and pressure(1,2,3). The following studies were undertaken to examine any possible effect of acetazolamide on passage of protein from CSF to plasma in dogs.

Methods. Mongrel dogs of both sexes, anesthetized with a combination of morphine and sodium pentobarbital, were used. The dogs were continued under the pentobarbital anesthesia throughout the experiment. These dogs were divided into 2 groups. The first group was a series of 8 dogs ranging in weight from 10 kg to 19.5 kg. Each dog was given $\frac{1}{2}$ ml (25 μ c) of radioiodinated human serum albumin (RISA-Abbott) intrathecally and 35 mg acetazolamide per kg intravenously. In addition, ureters of 3 of these 8 dogs were ligated and cut close to the kidney.

Passage of the tagged albumin from CSF into the bloodstream was then measured in controls and experimental group over a 24 hour period by monitoring the plasma for radioactivity according to a technic described previously(4). Radioactivity in the CSF at the end of 24 hours was also determined. In the 3 dogs with tied ureters the ligatures were inspected at end of experiment.

A second group of 6 dogs ranging in weight from 12.4 kg to 15.0 kg was studied by a different technic. Two ml of CSF from each dog was withdrawn from the cisterna magna and replaced with 2 ml of freshly-drawn, heparinized plasma taken from the same dog. Three of the 6 dogs received intravenous acetazolamide in a dose of 35 mg/kg at time of

intrathecal plasma injection. Each of the 6 dogs was then tilted alternately head up and head down for 2 hours after which 1 ml CSF sample was taken. Total protein determinations were made on all CSF samples. Data and samples from dogs not yielding 3 successive clear CSF specimens were discarded.

Results. Data from the first group of 8 dogs indicate that acetazolamide appreciably slows normal passage of albumin from the CSF to the bloodstream. Fig. 1 shows the percentage of intrathecally injected albumin which could be demonstrated in the bloodstream at various intervals. Previously published data from 12 control dogs are also presented.

Less than half the normal amount of intrathecally injected radioactivity can be accounted for in the bloodstream after 24 hours in the dogs given acetazolamide. Data from the dogs with tied ureters showed that this decreased appearance was not an artefact due to increased urine output. The dogs with tied ureters showed even less of the intrathecally injected radioactivity in the bloodstream than those with intact ureters, but the difference is probably not significant. In the dogs with tied ureters the ligatures were seen to be intact with negligible hydroureter at end of experiment.

A comparison between amount of radioactivity left in the CSF of the control dogs and dogs given acetazolamide was made at end of experiment. A value representing total radioactivity remaining in the CSF for each dog was obtained by multiplying the counts per ml of CSF at end of experiment by the CSF volume, which was assumed to be proportional to the weight of the dog and arbitrarily set at 1 ml per kg. Thus, without knowing the exact percentage of injected radioactivity remaining in the CSF at the end of 24 hours, one could still compare the 2 groups. The dogs receiving acetazolamide were found to have from 3 to 7 times as

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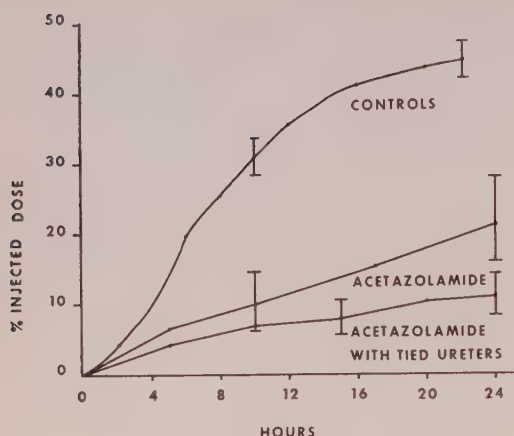


FIG. 1. Plasma radioactivity after intrathecal inj. of RISA in control dogs and dogs given a single I.V. dose of acetazolamide (35 mg/kg) simultaneously. Values represent % of intrathecal dose demonstrable in plasma $\pm$ stand. error.

much radioactivity in the CSF when compared with control dogs.

The pre-injection CSF protein level in a second group of 6 dogs was about 35 mg%. Two hours after intrathecal plasma injection the values were raised to an average of 1,000 mg% in both the untreated group and in the group receiving acetazolamide. Ten hours after injection, CSF protein levels in 3 dogs that did not receive the drug had returned to an average value of 166 mg%, while the 3 dogs given acetazolamide had average values of 875 mg%.

Discussion. We have concluded that acetazolamide slows the movement of protein from CSF to the bloodstream in the dog because bloodstream radioactivity after intrathecal RISA was diminished in dogs given acetazolamide. In addition, acetazolamide causes increased retention of CSF radioactivity in dogs given intrathecal RISA. And finally, acetazolamide retards the return to normal of artificially elevated CSF proteins. Precise explanation of the mechanisms involved in this slowing is unavailable.

The only known primary effect of acetazolamide is its ability to inhibit carbonic anhydrase(3,5). Any observed effect of acetazolamide should therefore be explained on the basis of carbonic anhydrase inhibition. Conversely, a demonstrable effect of acetazolamide on a system is presumptive evidence

that the system contains carbonic anhydrase.

The assumption that carbonic anhydrase is involved in an active transport of protein from the CSF would explain the experimental results reported here. Since there is no direct evidence to support this assumption, we chose to explain our experimental findings in another manner.

Reduction by acetazolamide of CSF pressure and flow in animals has been reported(2,6). However, other studies have not been in complete agreement with these findings(7). Decrease of CSF pressures in children(1), and reduction in frequency of CSF aspiration by acetazolamide therapy in a case of hydrocephalus have also been reported(8). It has been suggested that inhibition of carbonic anhydrase at the choroid plexus and other possible secretory sites decreases the flow of CSF which is secondary to inhibition of an active chloride transport system from plasma to CSF(3).

Our interpretation is that the reduction by acetazolamide in rate of movement of protein from the CSF is secondary to a reduction in CSF flow which is necessary to keep the protein in contact with the sites of absorption. The sites of absorption are probably similar to those demonstrated in the cat(9).

Summary. Acetazolamide was shown to inhibit the passage of protein from CSF to plasma in dogs. This action was demonstrated with both normal and abnormally-elevated CSF protein levels.

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Alloxan Diabetes and Insulin Effects on Amino Acid Incorporating Activity of Rat Liver Microsomes.* (26255)

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In alloxan-diabetic rats there is a progressive decrease in peptide-synthesizing capacity of the liver(1,2). The specific question to be tested here was whether any component of the microsomal peptide synthesizing system(3-5) could be shown to be altered in activity by lack or excess of insulin in the liver donor.

Materials and methods. Two types of experiments were done. In one type microsomes were prepared from 300 g male Sprague-Dawley diabetic rats previously given alloxan and maintained on insulin 3 weeks(6); during this period each rat was removed once from insulin and food for 24 hours and its fasting blood sugar determined; those with values above 300 mg% were selected. Seventy-two hours before use diabetic rats were separated into 2 groups: one group was continued on insulin sufficient to prevent glycosuria (control diabetics); the other received no insulin and developed hyperglycemia and glycosuria (untreated diabetics); both were fasted in metabolism cages 24 hours before use. Urinary ketones were estimated semi-quantitatively as acetoacetic acid(7). At sacrifice, blood sugars(8,9) were estimated on tail vein specimens; all untreated diabetics had fasting blood sugars above 300 mg%. In the second type of experiment, normal 300 g rats were treated with excess insulin: 8 units protamine-zinc-insulin (Lilly) and 4 units regular insulin at 20 hours, and again with 2 units regular insulin at 4 hours, before sacrifice; these animals were fasted 24 hours before use.

The microsomes, the supernatant fraction, and the fraction containing amino acid acti-

vating enzymes (precipitated at pH 5 from the supernatant fraction and redissolved at pH 7.6) were prepared according to Zamecnik *et al.*(3,4,10,11), with 0.35 M sucrose-0.05 M Tris buffer, pH 7.6, as the homogenizing medium. The microsome fraction from each liver was resuspended in the buffered sucrose; its protein concentration was estimated(12) and adjusted by dilution so that equal amounts of microsome protein could be incubated; aliquots were also analysed for protein nitrogen, phospholipid phosphate and ribonucleic acid(13). A stock pH 5 enzyme preparation from normal liver was used throughout, unless otherwise specified. Microsome fractions were incubated in triplicate 20 minutes at 37°C as shown in Tables I and IV, after which ice-cold 10% trichloroacetic acid was added. Protein was then isolated and its radioactivity estimated(14). The concentration above which there was no further increase in incorporation was ascertained for each component of the medium, and each was subsequently used at a concentration above this to insure that only the activity of the microsomes would be rate-limiting.

Results. Control experiments indicated that incorporation of leucine-1-C¹⁴ into microsomal protein, as here measured, was an enzyme catalysed process: (a) no incorporation occurred up to one hour at 2°C, nor at zero time at 37°C; (b) incorporation increased linearly with time up to one hour at 37°C; (c) ATP (adenosinetriphosphate), GTP (guanosine triphosphate), an ATP-generating system (*e.g.*, phosphoenol pyruvate and pyruvate kinase), and a fraction of soluble liver protein were required; (d) incorporating activity of microsomes and activating activity of the soluble fraction were lost by heating either fraction at 55°C for 5 minutes; (e) no incorporation occurred when pH 5 enzyme fraction was incubated without microsomes.

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Diabetic animals. In each of 2 experiments microsomes from livers of untreated diabetic rats were less active in amino acid incorporation than those from control (insulin treated) diabetics (Table I). In both series loss of amino acid incorporating activity in microsomes was associated with development of ketonuria in the untreated diabetic rats (Table I). No difference was found with respect to ability of soluble fractions to activate microsomal incorporation (Table II).

In microsomes from untreated diabetic rats

TABLE I. Effects of Insulin Withdrawal from Alloxan-Diabetic Rats on Amino Acid Incorporating Activity of Liver Microsomes: Group 1, Maintained on Insulin (See Text); Group 2, Insulin Withdrawn for 72 Hr. Each incubation sample in Exp. 1 contained 5 mg microsomal protein, 1.5 mg pH 5 enzyme protein from normal rat liver, 0.02 mg pyruvate kinase protein, 10 μ moles phosphoenol pyruvate, 1.0 μ mole ATP, 0.25 μ mole GTP, 5 μ moles Mg^{++} ion, and 0.9 μ mole DL-leucine-1- C^{14} with 4.5×10^6 cpm in 1 ml incubation medium. Conditions were the same in Exp. 2, except that 6 mg microsomal protein was used.

Group	Rat No.	Blood sugar, mg %	Urinary ketones, %	Mean specific radioactivity of microsomal protein, cpm/mg
Exp. 1				
1	1	Not measured	0	381
	2		0	346
	3		0	321
	4		0	314
	5		0	307
	6		0	276
Mean				$324 \pm 15^*$
2	1	404	10	326
	2	364	10	312
	3	364	30	257
	4	360	30	234
	5	380	30	187
	6	384	80	172
Mean				$248 \pm 26^*$
Exp. 2				
1	1	Not measured	0	303
	2		0	279
	3		0	276
	4		0	273
	5		0	226
Mean				$271 \pm 13^*$
2	1	312	10	212
	2	316	80	190
	3	376	80	167
	4	368	300	145
	5	640	300	113
Mean				$165 \pm 17^*$

* Stand. error. Difference between Groups 1 and 2 is significant to $P < 0.02$ in Exp. 1 and $P < 0.001$ in Exp. 2.

TABLE II. Comparison of Activating Effects of Pooled Soluble Fractions from 6 Normal Rats and 6 Untreated Diabetic Rats (Group 2, Exp. 1, Table I). Each incubation sample contained: 6.0 mg normal microsomal protein, 3.0 mg pooled soluble liver protein, 20 μ mole creatine phosphate, 1.0 μ mole ATP, 0.25 μ mole GTP, 5.0 μ mole Mg^{++} , and 0.5 μ mole DL-leucine in 1 ml.

Normal microsome donor No.	Mean specific radioactivity of microsomal protein, cpm/mg	
	With soluble fraction from Normals	Diabetics
1	134	150
2	144	156
3	154	172
4	186	175
5	185	185
6	206	186
Mean	$167 \pm 11^*$	171 ± 6

* Stand. error. Difference between means is not significant.

there was a decrease in ratio of phospholipid phosphate to protein nitrogen, and a slight increase in ratio of RNA to protein nitrogen relative to control diabetics (Table III). This suggests that in the diabetic animal the synthesis of endoplasmic reticulum lipoprotein may be depressed.

Excess insulin. Insulin treatment of normal rats prior to sacrifice resulted in increased amino acid incorporating activity of the liver microsomal fraction (Table IV). Absolute values in Table IV cannot be compared with those of Table I because the whole soluble supernatant fraction was used as activator in Table IV, and the pH 5 fraction in Table I.

No effects of insulin incubated in the medium with normal liver microsomes could be demonstrated, the mean values for 4 experiments without and with 0.01 unit insulin (Lilly crystalline, lot 657501) per ml being 136 ± 13 and 132 ± 7 cpm/mg microsomal protein, respectively. The incubation conditions were the same as for the normal series of Table II except that 6.5 mg normal liver microsomal protein were used in each vessel.

Discussion. Impaired amino acid incorporation into microsomes from diabetics, and the increased incorporation into microsomes from insulin-treated normal animals, appears to represent some change of enzyme activity of the microsomal fraction. These variations

TABLE III. Composition of Microsomes from Alloxan-Diabetic Rats: Group 1, Maintained on Insulin (See Text); Group 2, Insulin Withdrawn for 72 Hr. Diabetic animals are the same as those of Table I, Exp. 2. Values for normal animals are those of Palade and Siekevitz(13).

Group	mg RNA	μg phospholipid P
	mg protein N	mg protein N
1	.84 ± .058*	152 ± 10*
2	1.09 ± .045*	110 ± 6*
Normal rats	1.12	157
	.91	152
	.99	133

* Mean ± stand. error, 5 rats/group. Differences between Groups 1 and 2 are significant to $P < 0.007$ for each ratio.

do not stem from variations in size of a free leucine pool, as the microsome pellets were well washed; neither are they the consequence of differences in activity of soluble protein or pH 5 fraction, as these were prepared from normal liver and equal amounts were added to all incubation samples in a given experiment.

The correlation of impairment of amino acid incorporating activity with increasing ketosis in the liver donor suggests that capacity of the liver to metabolize fat normally and ability to synthesize protein fail concurrently. Whether or not there is a causal re-

TABLE IV. Effect of Insulin Treatment of Normal Rats on Amino Acid Incorporating Activity of Liver Microsomes: Group 1, Normal Controls; Group 2, Insulin Treated (See Text). Each incubation sample contained 5 mg microsomal protein, 4 mg soluble protein from normal liver, 1 μmole ATP, 0.025 μmole GTP, 5 μmoles Mg^{++} ion, and 0.9 μmole DL-leucine-1- C^{14} with 4.5×10^6 cpm in 1 ml medium.

Group	Rat No.	Mean specific radioactivity of microsomal protein, cpm/mg
1	1	115
	2	98
	3	96
	4	88
	Mean	99 ± 6*
2	1	181
	2	156
	3	148
	4	133
	Mean	155 ± 10*

* Mean ± stand. error. Difference between Groups 1 and 2 is significant to $P < 0.002$.

lation between these 2 defects remains to be investigated.

After the present study was initiated, Korner(15) demonstrated a decreased ability of liver microsomes from hypophysectomized rats to incorporate labeled amino acids into protein. Normal incorporating activity was restored by administration of insulin prior to removal of the tissue. Doell(16) reported a 40 to 50% increase in amino acid incorporating activity of microsomes after injection of insulin into normal non-fasting rats.

Summary. (1) Liver microsomes from severely diabetic rats incorporate leucine-1- C^{14} into protein at a lower rate than microsomes from diabetic control rats maintained on insulin. The former have also a lower phospholipid-P and a higher RNA content per unit of protein-N. (2) Microsomes from normal rats incorporate amino acids at a higher rate after injection of insulin. (3) These changes occurred specifically in microsomes; no difference was found with respect to ability of soluble fractions to activate microsomal incorporation. Insulin was without effect upon the microsomal system *in vitro*.

ADDENDUM: Korner(17) found amino acid incorporating capacity of liver microsomes from a different type of diabetic rat to be defective.

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A Non-Hydrazine Monoamine Oxidase Inhibitor with Antihypertensive Properties. (26256)

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Previous studies in this laboratory have suggested that effective inhibition of monoamine oxidase (MAO) by various drugs in human hypertensives results in an orthostatic decrease in blood pressure(1-3). Agents of this type include iproniazid (Marsilid), trans - DL - 2-phenylcyclopropylamine (SKF-385, Parnate), β -phenylisopropylhydrazine (JB-516, Catron), phenylzine (Nardil), nialamide (Niamid), DL-serine-N²-isopropylhydrazide (RO-4-1038)(4), and isocarboxazid (Marplan)(5). However, any final conclusion concerning a relationship between enzyme inhibition and blood pressure lowering has been tempered by the fact that, with the exception of SKF-385, all of these agents are hydrazine derivatives. Also, either because of low potency or occurrence of prohibitive toxicity, none have become valuable therapeutic agents in hypertension.

Thus, the discovery of the potent, non-hydrazine, MAO inhibitor, N-benzyl-N-methyl-2-propynylamine·HCl (MO-911), by Taylor *et al.*(6) was of special interest. This report presents initial results of administering this compound to human subjects. The findings give added support to the hypothesis that pronounced MAO inhibition produces an orthostatic decrease in blood pressure in hypertensive patients. Barring unforeseen toxicity, MO-911 appears to be a promising antihypertensive agent.

Materials and methods. The subjects were 4 females and 5 males, aged 26 to 51 years, with sustained hypertension uncomplicated except for renal insufficiency (pt. C.F.), polycystic kidneys (pt. C.M.) and unilateral pyelonephritis (pt. W.C.). Except for occasional one- to 3-day passes, the subjects

were hospitalized throughout the periods of study. MO-911 was supplied as 25 mg scored tablets.* The only other medications were cardiac glycosides, barbiturates for sleep and salicylates. Salt intake was moderately restricted in one patient and calories in another.

Blood pressures (arm cuff technic) and pulse rates were measured 4 times daily in the supine position and also after 2 minutes of quiet standing. Control observations were made over a period of at least 6 days. Then, MO-911 was administered orally as a single dose before breakfast each day for periods of 14 to 34 days. Initial dosage was 75 to 125 mg/day with a downward revision when pronounced decline of blood pressure developed. With one exception, observations were extended for 8 to 17 days after cessation of therapy; during this time blood pressure levels approached or had returned to pre-treatment values.

Serial blood counts, urinalyses, tests of renal and hepatic function and periodic screening of visual acuity and color vision were performed to detect possible toxic effects. Degree of MAO inhibition was evaluated by daily assays of urinary tryptamine and tyramine(3).

Results. It was quickly established that MO-911 is an effective antihypertensive agent as well as a potent MAO inhibitor. Its long duration of action permitted achievement of sustained effects with use of single daily doses.

Effects on blood pressure and pulse rate. A pronounced fall in standing blood pressure

* Through the courtesy of Dr. R. K. Richards, Abbott Laboratories, N. Chicago, Ill.

TABLE I. Blood Pressure Response to MO-911.

Patient	Dose, mg/day	Avg blood pressures (mm Hg)*					
		Control		On drug		Change	
		Supine	Standing	Supine	Standing	Supine	Standing
C.F.	37.5- 75	219/143	224/151	218/128	130/ 93	- 1/-15	-94/-58
W.C.T.	50 - 75	161/109	148/107	165/110	117/ 81	+ 4/+ 1	-31/-26
E.T.	75 -100	204/122	202/126	190/123	136/ 93	-14/+ 1	-66/-33
R.U.	50 -100	177/117	158/119	177/114	106/ 80	0/- 3	-52/-39
E.M.	50 - 75	203/124	181/123	174/102	118/ 84	-29/-22	-63/-39
C.M.	50 -125	164/117	156/121	192/120	138/108	+28/+ 3	-18/-13
M.K.	50 -125	213/146	188/149	205/135	129/106	- 8/-11	-59/-43
W.C.	50 - 75	220/137	211/149	222/134	148/108	+ 2/- 3	-63/-41
R.L.	25 - 75	154/113	148/120	169/120	124/ 98	+15/+ 7	-24/-22

* Each value is avg of 20 measurements in a period of 5 days prior to therapy (control) or at peak of drug effect (on drug).

occurred in each patient, average decreases ranging from 18 to 94 mm Hg in systolic and 13 to 58 mm Hg in diastolic pressure (Table I). In contrast, supine pressures were usually unchanged from control values. Time of onset for a pronounced orthostatic effect with doses of 75 to 125 mg/day varied from 7 to 20 days. After discontinuation of the drug, an orthostatic effect was usually discernible for 2 weeks or longer. Some of these relationships are illustrated by the findings in a typical case (pt. R.U.) (Fig. 1).

The patients did not develop tachycardia

even in presence of profound decreases in standing blood pressure. Instead, alterations in pulse rate with posture were approximately the same as seen in the control state, with increases on standing of up to 18 beats per minute.

MAO inhibition. Marked inhibition was shown by 4 to 11-fold rises in urinary tryptamine and similar changes in tyramine output, except in patient C.F. whose tryptamine rose from 65 to 220 $\mu\text{g}/\text{day}$; the lessened chemical response observed in this case is attributable to renal insufficiency (creatinine clearance of 50 ml/min). The tryptamine response shown in Fig. 1 indicates the usual magnitude of effect and general correlation with blood pressure changes.

Other effects. All patients maintained a sense of well being in spite of the marked alterations in blood pressure. Three showed heightened mood, 3 exhibited drowsiness early in treatment, one had slight morning dryness of the mouth, 2 had mild constipation, one had a disturbed sleep pattern, one had transient paresthesias, and one experienced an inability to ejaculate. Orthostatic dizziness occurred in some patients but could be obviated by a decrease in dose. There were no obvious changes in appetite. The aforementioned symptoms disappeared during the post-treatment period.

No abnormalities were noted in serial blood counts or in the results of tests of liver, renal and visual functions.

Discussion. This demonstration of the antihypertensive properties of MO-911, whose

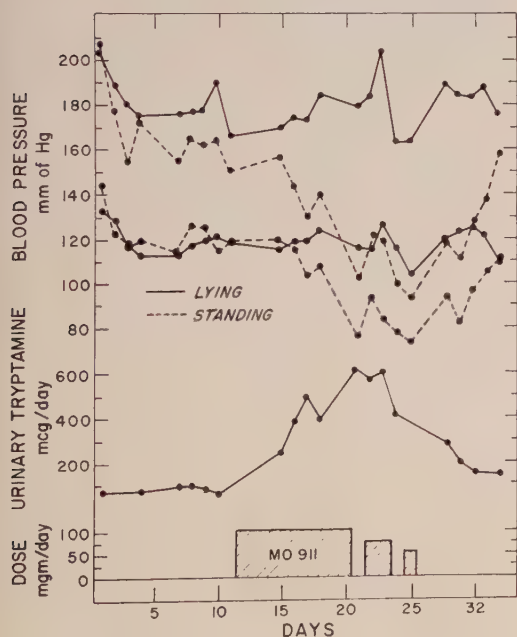


FIG. 1. Example of changes in blood pressure and urinary tryptamine with MO-911 (pt. R.U.).

structure differs radically from that of previous MAO inhibitors, provides apparently conclusive evidence of a relationship between MAO inhibition and lowering of blood pressure. It is likely that the site of action is in the sympathetic nervous system since the blood pressure change is predominantly orthostatic and occurs in absence of reflex tachycardia or marked disturbance in parasympathetic functions. That specific sympathetic block may occur is indicated by the findings of Gertner(7) that several MAO inhibitors produced blockade of transmission through the perfused superior cervical ganglion of the cat, without interruption of responses to acetylcholine. Evidence of selective inhibition of sympathetic ganglia in the intact animal has been obtained by Goldberg and DaCosta(8). Assuming that the sympathetic block is due to MAO inhibition, mediation might be *via* increased tissue levels of amine(s). Norepinephrine must logically receive prime consideration and actually is known to have ganglion blocking properties (9). In support, Spector *et al.*(10) have recently reported that MAO inhibition results in increased levels of norepinephrine in the sympathetic ganglia of rabbits.

Much attention is now being given to therapeutic use of guanethidine (Ismelin) and bretylium tosylate (Darenthin)(11), agents which produce orthostatic hypotension *via* sympathetic blockade in absence of parasympathetic blockade. Our preliminary findings suggest that MO-911 compares favorably with these drugs and may also have desirable psychic properties. The drug is sufficiently potent to be used alone, although adjuvant agents could reasonably be expected to reduce dose requirements and might improve the responsiveness of some patients. The slow onset and long duration of its action are ideally suited to management of the ambulatory hypertensive. For initiation of treatment daily doses of 100 to 125 mg given under close observation are recommended. When the desired blood pressure effect has been established, the dose can be reduced to maintenance levels which will probably range

from 37.5 to 75 mg/day. Alternatively one might begin treatment at 37.5 mg/day and increase the daily dose 12.5 mg every 2 weeks until optimal effect is obtained.

The effectiveness of MO-911 as an anti-hypertensive agent is considered established. From data in laboratory animals(6), it would appear to have a more favorable therapeutic index than has been available heretofore with MAO inhibitors. However, the question of chronic toxicity, particularly hepatic and neurologic reactions as encountered in some patients receiving hydrazine-containing inhibitors, can be resolved only by further clinical trial.

Summary. The compound, N-benzyl-N-methyl-2-propynylamine · HCl (MO-911), is shown to be a potent monoamine oxidase inhibitor in man, with hypotensive properties. Since its structure is different from that of previous inhibitors, the findings support the hypothesis that effective inhibition of monoamine oxidase results in orthostatic lowering of blood pressure, presumably by selective decrease of sympathetic nerve activity. Extensive clinical trial of the drug as an anti-hypertensive agent is warranted.

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Connective Tissue IV. Synthesis and Turnover of Proteins in Tissues of Rats.* (26257)

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Neuberger *et al.*(1) reported that collagen of rat tendon is metabolically relatively inert. Harkness(2) reported that collagen disappears rapidly during the postpartum involution of rat uterus. Gould(3) found that collagenolysis appears in scorbutic guinea pigs. The present study is undertaken to determine the synthesis and turnover rates of collagen and elastin in various tissues.

Methods. Female rats, Wistar strain, 5 weeks of age were injected, intraperitoneally, with saline containing 7.8 μ c of uniformly labeled C^{14} -lysine[†]/100 g body weight. Groups of 3-6 rats were sacrificed at 1, 4, 8 and 24 hours, 3, 10, 30, 40, 60 and 90 days after C^{14} -lysine had been administered. Tissue samples (0.1-0.5 g) from aorta, uterus, tendon and skin were homogenized with 10 ml normal saline in a VirTis "45"[‡] homogenizer for 5 minutes at 50 setting. The homogenate was centrifuged at $27,000 \times g$ for 30 minutes. The residue was extracted with 16 ml 0.1N NaOH at room temperature overnight and centrifuged at $105,000 \times g$ in a Spinco Model L centrifuge for 1 hour. The resulting residue was extracted again with 10 ml 0.1N NaOH at room temperature for 2 hours. The supernatants of the saline and the 2 base extractions were combined, dialyzed against distilled water and evaporated to dryness. Soluble collagen (SC) was extracted from the dried specimen with 0.3 M trichloroacetic acid (TCA) at 90°C (4). The residue from this extraction was nonscleroprotein (NSP).

The insoluble collagen (IC) was extracted from the basic residues mentioned above by use of 0.1N NaOH at 100°C for 15 minutes. This extraction was repeated. The 2 extracts

thus obtained were combined and dialyzed against distilled water. The final residue was elastin (E). Proteins of each fraction, except in the case of E, for which, in tendon, skin and uterus, the amount was too low, were hydrolyzed in 6N HCl in a sealed tube. Nitrogen was determined by Conway micro-diffusion method(5). Protein was calculated as $6.25 \times \text{N}$. Aliquots of the hydrolyzate were evaporated to dryness, neutralized and made up to a concentration of 0.5-1.0 mg protein/ml. One ml of the neutralized hydrolyzate was evaporated to dryness on a stainless steel planchette for radioactivity determination. Isotope counting was done by an automatic gas flow counter with a counting error of 2.5%. Half-lives were calculated according to Radiation Health Handbook and Glasstone(6,7).

Representative samples of soluble and insoluble collagen, respectively, were subjected to treatment by column chromatography according to the method of Moore and Stein (8). Chemical and radioactivity determinations were carried out by the method of Kao *et al.*(9).

Results. The synthesis and turnover of proteins in aorta of rat are shown in Fig. 1. Specific activity (SA) (cpm/mg of protein) of NSP reached its maximum, 220, at 24 hours and decayed with a half-life of 14 days. The SA of SC reached its maximum, 140, at 24 hours and decayed with a half-life of 14 days. The SA of IC reached its maximum, 42, at 10 days. From 10 to 40 days the SA decreased with a calculated half-life of 15 days. Between the 40th and 90th day there was no further decrease in the SA of IC. The SA of E reached its maximum, 33, at 30 days and decayed with a half-life of 53 days. The synthesis and turnover of proteins in uterus is shown in Fig. 2. Uterine protein fractions had highest SA of the tissues studied. The NSP reached its maximum, 400, at 4 hours

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[†] Purchased from Nuclear Chicago, Inc.

[‡] VirTis Co., Yonkers, N. Y.

and decayed with a half-life of 12 days. The SC reached its maximum, 260, at 8 hours and decayed with a half-life of 12 days. The IC

reached its maximum, 100, at 3 days and decreased up to 30 days with a half-life of 11 days. There was little decay in activity

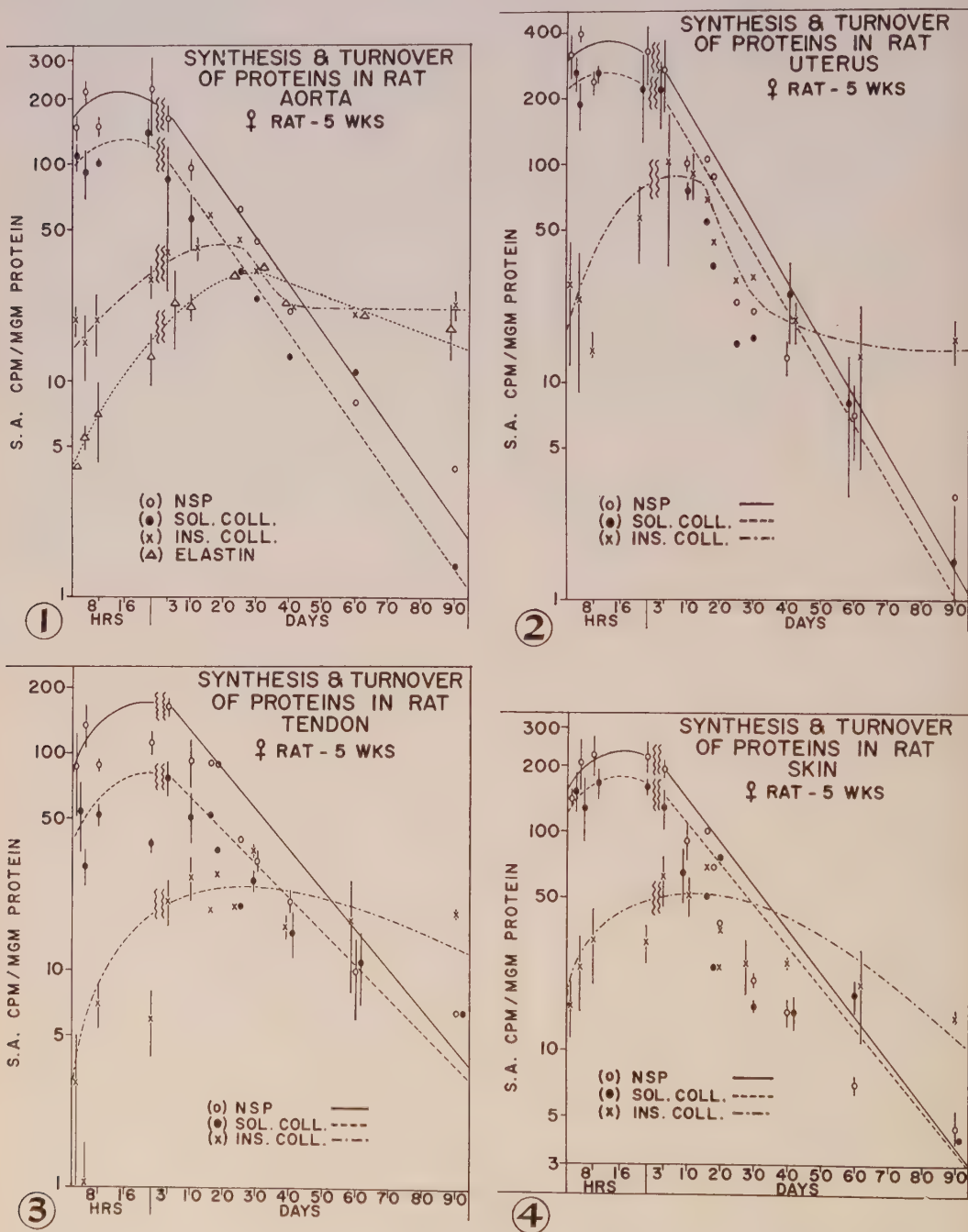


FIG. 1. Synthesis and turnover proteins in rat aorta. All bars represent stand. dev. All symbols represent means.

FIG. 2. Synthesis and turnover proteins in rat uterus (see legend for Fig. 1).

FIG. 3. Synthesis and turnover proteins in rat tendon (see legend for Fig. 1).

FIG. 4. Synthesis and turnover proteins in rat skin (see legend for Fig. 1).

TABLE I. Synthesis and Turnover of Insoluble Collagen and Elastin after Correction for Growth (SA = cpm/mg Protein).

		Days after C ¹⁴ -lysine injection					
		3	10	30	40	60	90
		Actual value of SA					
Insoluble collagen—	Aorta	38	41	32	22	20	22.5
	Uterus	101	89	30	19	13	15.5
	Tendon	21	27	36	16	17	18.5
	Skin	61	51	25	25	20	14.5
Elastin—	Aorta	23	22	33	23	20	17.5
		SA corrected for weight					
Ratio	Body wt (W)	max	1.51	2.16	2.20	2.65	3.12
	Body wt at max SA (W max)	max	max	1.43	1.46	1.76	2.07
			max	max	1.01	1.23	1.44
Insoluble collagen—	Aorta		41	46	32	35	46
	Uterus	101	134	65	42	34	48
	Tendon			36	16	21	27
	Skin	61	77	54	55	53	45
Elastin—	Aorta			33	23	24	25

thereafter. The synthesis and turnover of protein in tendon is shown in Fig. 3. The NSP reached its maximum, 160, at 3 days and decayed with a half-life of 16 days. The SC reached its maximum, 80, at 3 days and decayed with a half-life of 20 days. The IC reached its maximum, 25, at 10 days and decayed with a half-life of 65 days. The synthesis and turnover of protein in skin is shown in Fig. 4. The NSP reached its maximum, 220, at 8 hours and decayed with a half-life of 15 days. The SC reached its maximum, 170, at 8 hours and decayed with a half-life of 17 days. The IC reached its maximum, 52, at 3 days and decayed with a half-life of 28 days.

Discussion. These data indicate that soluble collagen has higher specific activity and higher turnover rate than insoluble collagen and elastin. By column chromatography and subsequent counting, it was shown that specific activity of lysine and hydroxylysine is higher in soluble than in insoluble collagen. This agrees with reports of other workers (10, 11, 12, 13, 14) that soluble collagen is the precursor of insoluble collagen.

From the present data it is also shown that there is a difference in rate of synthesis and turnover of insoluble collagen in various organs. In the tissues studied, the collagen of uterus had the highest rate of synthesis, highest specific activity, as well as shortest half-

life. By contrast, the collagen of tendon had the lowest rate of synthesis and lowest specific activity. Collagen turned over more slowly in the aorta than in any other tissue, if, indeed, it turned over at all.

Since rats used in this experiment were 5 weeks of age, rate of decay was influenced by new collagen formed after C¹⁴-lysine had been administered. Therefore, insoluble collagen and elastin were actually metabolized more slowly than indicated by the data shown in the figures. In Table I, the data have been corrected on the assumption that new formation of collagen and elastin was directly proportional to increase in body weight. From this table, it is clear that there is a turnover of insoluble collagen and elastin in connective tissue and that while the "dilution factor of new tissue" is important, it does not alone account for all of the changes observed.

Summary. 1. The synthesis and turnover of protein in aorta, uterus, tendon and skin were studied in 5-week old rats. 2. The SA of connective tissue proteins in decreasing order of magnitude was nonscleroprotein, soluble collagen, insoluble collagen and elastin. 3. Among the tissues studied, greatest synthesis and turnover of collagen was observed in uterus. As regards synthesis, skin, aorta and tendon followed in descending order, while, for turnover, the order was skin, tendon and aorta.

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Metabolism of Exogenous Sialic Acid in the Rat.* (26258)

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The ubiquitous occurrence of sialic acid in mammalian tissues as a component of glycoproteins, glycolipids and oligosaccharides(1) implicates a role for this acidic carbohydrate in a variety of basic physiological phenomena. Free, unbound, sialic acid has not been detected except in cerebrospinal fluid (2). The liberation of sialic acid by bacterial and viral neuraminidase(3) raises the question of the metabolic fate of this substance. This report is concerned with the metabolism of sialic acid following intra-arterial, intraperitoneal, subcutaneous and oral administration to the rat.

Materials and methods. Sialic acid was prepared from bovine submaxillary mucin by the method of Svennerholm(4). The lyophilized product obtained following chromatography on Dowex-1 was dissolved in 0.9% saline and neutralized in preparation for administration to the animals. Twice recrys-

tallized material (m.p. 185-186°C), which was shown by paper chromatography(5,6) to contain N-acetylneuraminic acid and N-glycolylneuraminic acid in the approximate ratio of 9:1, was used for calculation of extinction coefficients with the thiobarbituric acid assay(7).

Adult male albino rats were anesthetized with chlorolose-urethane and heparinized. The abdominal aorta was cannulated with a T-cannula and sialic acid (approximately 100 mg/kg body wt, in 1.0 ml) was injected through the side arm followed by 1.0 ml of saline. Arterial blood samples were obtained at time intervals indicated and diluted plasma samples were analyzed for free sialic acid using the thiobarbituric acid assay of Warren(7). In several experiments the renal arteries were bilaterally ligated. Data from typical experiments are presented in Table I.

The assay of sialic acid in urine was complicated by the presence of a chromogen resembling that given by deoxyribose. How-

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ever, satisfactory results were not obtained with calculations based on the extinction coefficients of deoxyribose and sialic acid. Rat urine, chromatographed on Whatman No. 1 paper in *n*-butanol, acetic acid, water (4:1:5, v/v) and developed with the thiobarbituric acid spray reagent(8), showed a spot with a lower *R_f* than authentic deoxyribose. This chromogen was not identified, but interference was eliminated by use of the absorbancy ratio obtained with the thiobarbituric acid assay of control samples of rat urine. It was found that the ratio of optical densities ($O.D._{549}/O.D._{535}$) was equal to $0.71 \pm 0.06\sigma$. Since molecular extinction coefficients (ϵ) of sialic acid at 549 $m\mu$ and 535 $m\mu$ were found to be 55×10^3 and 26×10^3 respectively, and if it is assumed that the ϵ_{549} and ϵ_{535} of the urinary chromogen are 7×10^3 and 10×10^3 respectively, then equation (1) can be derived.

$$\mu\text{moles}_{S.A.} = 0.117 O.D._{549} - 0.082 O.D._{535} \quad (1)$$

It was shown that the recovery of various quantities (5 to 50 mg) of sialic acid added to 6 hour urine samples was $98.7\% \pm 1.2\sigma$ as calculated by equation (1). The measurement of free sialic acid excreted in the urine was carried out in a paraffinized metabolism cage. Six hour urine samples were collected and diluted to at least 100 ml for analysis. To determine the recovery of sialic acid added to the metabolism cage, 4.0 to 54.5 mg of sialic acid were pipetted into a cage with a rat. After 6 hours cage washings were analyzed with the thiobarbituric acid assay. Recovery of the added sialic acid was 95.4 to 99.0%. Antibiotic pretreatment of the gastrointestinal tracts of the rats was accomplished by adding phthalylsulfathiazole (sulfathalidine, Merck) to the rats' feed (10% by wt) and neomycin sulfate (Mycifradin sulfate, Upjohn) (0.25 g/100 ml) to the drinking water. This feeding was continued until the color of the feces turned from brown to gray or almost white. In experiments involving incubation of intestinal tissue with sialic acid, small strips (0.5-1.0 cm wide) were cut from the intestine and placed in 10 ml of Krebs-Ringer phosphate buffer(9) containing 0.4 mg of

TABLE I. Blood Levels of Free Sialic Acid with and without Renal Ligation.

Time after inj.	mg %/kg body wt	
	Rat 1,* without ligation	Rat 2,* with ligation
0 †	2.5	5.2
5 min.	160.4	74.2
30 "	19.4	43.0
1 hr	8.1	27.9
2 "	2.5	22.3
3 "	3.1	22.8
4 "	—	26.8
5 "	—	23.0
6 "	—	24.0

* Dosage of sialic acid—100 mg/kg body wt.

† Sample taken before inj.

either glucose or sialic acid and incubated at 37°C under an atmosphere of 95% O_2 /5% CO_2 in a Dubnoff metabolic incubator. Similarly, in experiments with intestinal contents, the cecum was washed out with buffer solution and 10.0 ml aliquots were incubated with known amounts of sialic acid in the Dubnoff incubator.

Results. Table I presents levels of free sialic acid in the rat blood following intra-arterial injection, with and without renal ligation. Without ligation all detectable sialic acid disappeared from the blood after 2 hours. With renal ligation the sialic acid content in blood reached a plateau after 2 hours, and no significant decrease in values was observed during the next 4 hours, the duration of experiment.

A large percentage of intraperitoneally administered sialic acid was excreted in the urine in the 0-6 hour period (Table II). The per cent recovery varied from about 80% with a 4.9 mg dose to approximately 90% with a 37.4 mg dose. Subcutaneous administration delayed excretion so that detectable amounts of sialic acid appeared in the 6-12 hour samples; however, the per cent recovery did not appear to differ from that of the intraperitoneal administrations of corresponding dosages. Fecal analyses for sialic acid did not reveal any detectable amounts of sialic acid in either of the parenteral administrations.

Sialic acid was not detected in the urine up to 24 hours following oral administration. The 2 rats receiving the largest doses of sialic

TABLE II. Recovery of Administered Sialic Acid from Rat Urine.

Route of admin.	Sialic acid admin. (mg/kg body wt)	Sialic acid recovered	
		mg/kg body wt	%
Intraper.	9.0	7.3	80.4
	18.2	14.5	80.0
	18.3	14.8	81.3
	37.1	31.8	85.8
	41.1	34.5	83.8
	68.0	62.4	91.7
Subcut.	41.6	34.8	83.7
	52.5	45.5	86.6
Oral (without antibiotic)	36.9	.0	.0
	46.7	.0	.0
	54.4	.0	.0
	75.5	.0	.0
	90.8	.0	.0
	273	.0	.0
Oral (with antibiotic pretreatment)	315	.0	.0
	90.0	.0	.0
	98.0	.0	.0
	182	.0	.0
	205	.0	.0

acid (Table II) were sacrificed after 12 hours and the intestines excised and washed out with saline solution. These washings did not contain a detectable amount of free sialic acid. Following antibiotic pretreatment of the gastrointestinal tract, orally administered sialic acid was not detected in any of the urine samples, but the feces was found to contain free sialic acid.

Enzymatic degradation of sialic acid by intestinal tissue slices could not be demonstrated. In every experiment in which tissue was incubated *in vitro*, the thiobarbituric acid assay showed a marked increase in optical densities with an absorption maximum at 535 m μ . This phenomenon has been previously reported(10). When intestinal contents were incubated with sialic acid, sialic acid had disappeared in both control and experimental solutions after 18 hours. However, this destruction of sialic acid was completely inhibited by addition of neomycin sulfate (125 μ g/ml) to the incubation solution.

Discussion. Following intra-arterial administration, sialic acid is rapidly cleared from the blood stream. If the renal arteries are tied off, equilibrium with the body fluids seems to be reached within 2 hours, since no

detectable decrease in the free sialic acid in the blood is noticeable during the next 4 hours. Thus, utilization is probably not a major factor in clearance of the material from the blood.

A large percentage (80-90%) of the sialic acid administered by intraperitoneal or subcutaneous injection is excreted in the urine during the first 6 hours after injection. The per cent recovery increases as dosage is increased, perhaps indicating saturation of the systems degrading sialic acid. Sialic acid was not detected in the urine or feces following oral administration of relatively large quantities. The presence of sialic acid in feces of rats whose gastrointestinal tracts had undergone extensive antibiotic treatment preceding oral administration indicated that intestinal bacteria are the principal cause of destruction of sialic acid. Furthermore, no evidence was obtained which indicates that intestinal tissue is capable of degrading sialic acid, and the disappearance of sialic acid when incubated in the presence of intestinal juice is inhibited by antibiotic. The fact that sialic acid did not appear in the urine of rats receiving antibiotics after oral administration may indicate that there is little or no absorption of sialic acid across the intestinal wall.

Summary. Sialic acid, prepared from beef submaxillary mucin and assayed by the thiobarbituric acid assay, was administered to rats intra-arterially, intraperitoneally, subcutaneously, and also orally with and without antibiotic pretreatment of the gastrointestinal tract. Cannulation experiments demonstrated the rapid removal of free sialic acid from the vascular system following intra-arterial administration. Analyses of 6 hour samples of rat urine following parenteral administrations showed that 80-92% of free sialic acid was recovered. Amount recovered increased as dose was increased. Intestinal bacteria destroyed the orally administered sialic acid which was not absorbed to any appreciable extent.

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Response of Starved Rats and Polycythemic Rats to Graded Doses of Erythropoietin.* (26259)

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Present studies of the humoral regulation of erythropoiesis are based on diverse *in vivo* bio-assay techniques, described by Gordon(1). Gordon also noted that methodology influenced results. Few specific attempts have been made to compare results obtained by different assay procedures(2,3).

This report details results of assays carried out using starved rats or rats rendered polycythemic by hypertransfusion. Reticulocyte counts and red cell Fe^{59} incorporation were measured; erythropoietic response of both types of assay animal to human and rabbit erythropoietin was assessed. Serially graded doses of erythropoietin were assayed and the relationship between response in red cell iron incorporation or reticulocyte production and dose of erythropoietin was determined.

Methods and materials. Erythropoietin was prepared from rabbit plasma and human urine: a) Hemolytic anemia was produced in albino rabbits by daily subcutaneous injections of 1 ml of 2.5% phenylhydrazine solution. When the hematocrits were 15% or less the rabbits were exsanguinated into heparinized flasks. The separated plasma was pooled, and extracted by the method of Borsook(4). This extract was concentrated 10-fold, dialyzed for 12 hours against distilled water at 4°C and stored frozen. The eryth-

ropoietic stimulating activity of such extracts had been previously demonstrated(4,5). b) Urine was obtained from a patient with severe anemia due to erythroid failure. His hematocrit ranged between 17 and 20%; there was persistent, absolute reticulocytopenia, and marrow was devoid of red cell precursors. Urine was dialyzed against running tap water for 18 hours, concentrated by flash evaporation to one-twentieth of the original volume and stored at -18°C.

Two types of bio-assay were used. On the first day of a 4-day assay period, female Sprague-Dawley rats weighing 180 to 200 g were started on starvation(6) or rendered polycythemic by a single transfusion of homologous packed red cells equivalent to 50% of the animals' original predicted red cell mass(7). Starvation was continued for the entire 96 hour assay period; the polycythemic animals were fed a regular rat chow diet. The remainder of the assay technic was standard. Plasma extract or urine concentrate was injected intravenously 30 and 54 hours after transfusion or institution of starvation. Although the dose of plasma extract or urine concentrate varied, volume of injection was kept constant by dilution with saline.

Eighteen hours prior to completion of the assay, each rat was given an intravenous injection of approximately 1 μC of Fe^{59} citrate in saline. An appropriate standard was also

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TABLE I. Hematocrit, Red Cell Fe⁵⁹ Incorporation, Reticulocyte Count and Total Red Cell Mass of Normal Rats and Assay Rats 96 Hours after Transfusion or Initiation of Starvation.

No. of rats	RBC Fe ⁵⁹ incorporation, %	Reticulocyte count, %	Hematocrit, %	Red cell mass,† cc/100 g
28 Normal	27.72 ± 6.19*	2.83 ± 1.15	44.28 ± 1.73	2.21
21 Starved	6.05 ± 1.77	.32 ± .08	52.85 ± 1.52	2.64
27 Polycythemic	5.00 ± 1.64	.38 ± .08	54.53 ± 2.59	2.83

* Stand. dev.

† Calculated with blood volumes described in reference 7 (normal rats) or text.

prepared. The assay was completed by determining red cell radio-iron incorporation, reticulocyte count and hematocrit of blood obtained from each rat by cardiac puncture at 96 hours.

Red cell radio-iron incorporation was determined by the following formula:

$$\% \text{ incorporation} = \frac{\text{Counts/ml blood} \times \text{total blood volume}}{\text{Total counts inj.}}$$

One ml of blood was counted in a well scintillation counter. Blood volume determinations by Fe⁵⁹ tagged homologous cells(8) on representative groups of assay rats revealed that the starved rats had a blood volume of $4.87 \pm 0.19\%$ (SD) of final body weight and the polycythemic animals $5.23 \pm 0.26\%$ (SD) of body weight. Hematocrits were determined by the micro technic. Reticulocyte counts were determined by the methylene blue technic(9). Number of reticulocytes per 1000 red blood cells were enumerated by 2 observers.

In all assays each dose of extract or concentrate was tested in 5 animals to obviate differences due to individual variation. Control values for iron incorporation, reticulocyte count and hematocrit were obtained for assay animals injected with saline. Results are recorded by plotting response in Fe⁵⁹ incorporation or reticulocyte level against the log of the dose of urine concentrate or plasma extract in milliliters. Regression lines(10) have been drawn for all sets of data and correlation coefficients(10) for the relation between dose and response have been determined.

Results. Table I records the hematocrit, reticulocyte count and red cell iron incorporation for both normal rats and saline injected control rats 96 hours after transfusion

or commencement of starvation. Both procedures produced elevation of hematocrit and a concomitant partial arrest of erythropoiesis, manifested by a fall in circulating reticulocyte count and iron incorporation.

The erythropoietic response of transfused assay rats to graded doses of erythropoietin obtained from both human urine and rabbit plasma is depicted in Fig. 1 and 2 respectively. In each instance iron incorporation increased as dose was raised; the log dose response relationships were linear and both showed high degrees of correlation. In contrast the changes in reticulocytes were more irregular, especially at lower dose levels; correlation was less than that seen for dose and response measured by Fe⁵⁹ incorporation.

The response of starved rats to rabbit plasma erythropoietin is shown in Fig. 3. The extract injected was prepared from a different pool of anemic rabbit plasma. The relationship between dose of rabbit erythropoietin and both iron incorporation and reticulocyte response was linear and high degrees of correlation were noted.

Finally, comparable doses of urine concentrate were assayed in starved rats or transfused rats to test the relative sensitivity of these 2 types of assay animals. Red cell radio-iron incorporation alone was measured and results are recorded in Table II. Regres-

TABLE II. Comparison of Erythropoietic Response of Polycythemic and Starved Rats Injected with Human Erythropoietin.

Dose in ml	Red cell Fe ⁵⁹ incorporation, %	
	Starved	Polycythemic
.050	9.3 ± 2.85*	10.2 ± 1.65
.100	10.9 ± 3.51	12.6 ± 2.31
.200	15.9 ± 4.61	14.7 ± 1.43
.500	23.9 ± 4.18	24.0 ± 2.58
1.000	22.9 ± .96	28.1 ± 1.56
2.000	31.1 ± 2.87	31.2 ± 3.48

* Stand. dev.

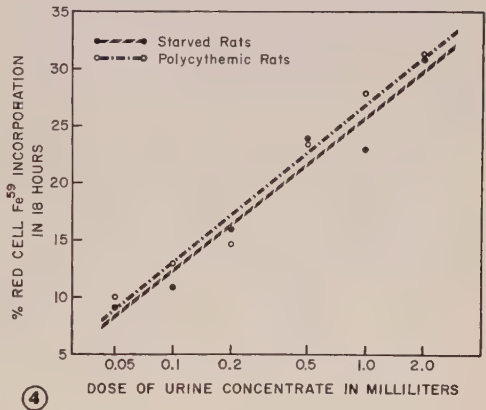
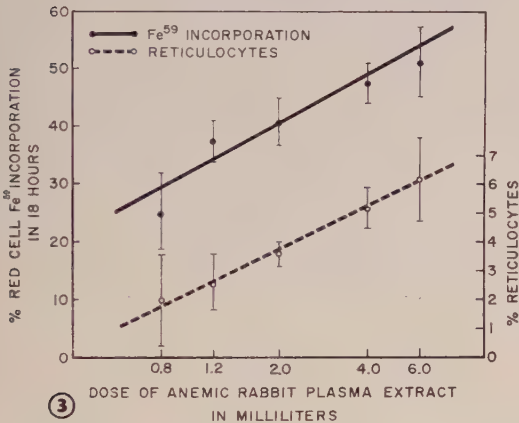
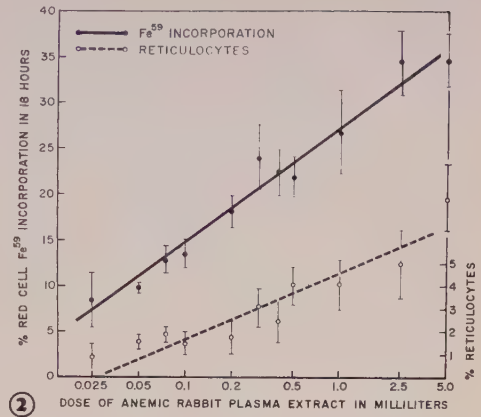
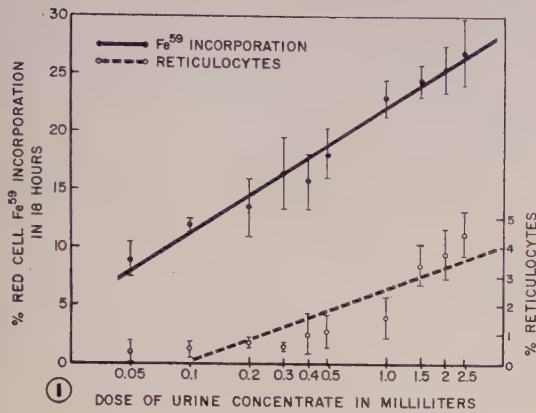


FIG. 1. Response of transfused rats to human erythropoietin. Each point represents mean ($\pm$ one S.D.) response of 5 assay animals. Correlation coefficients: dose-iron incorporation, .986; dose-reticulocytes, .884.

FIG. 2. Response of transfused rats to rabbit erythropoietin. Each point represents mean ($\pm$ one S.D.) response of 5 assay animals. Correlation coefficients: dose-iron incorporation, .983; dose-reticulocytes, .942.

FIG. 3. Response of starved rats to rabbit erythropoietin. Each point represents mean ($\pm$ one S.D.) response of 5 assay animals. Correlation coefficients: dose-iron incorporation, .964; dose-reticulocytes, .994.

FIG. 4. Response of starved rats and transfused rats to human erythropoietin. Each point represents mean response (S.D. recorded in Table II) of 5 assay animals. Correlation coefficients: starved rats, .974; transfused rats, .985.

sion lines for both dose-response relationships were constructed (Fig. 4). Results were similar for both types of assay animal.

Discussion. Few attempts have been made to compare the response of the starved rat to an erythropoietic stimulus with the response of rats in which erythropoiesis has been arrested by other means. Gurney and Pan(2) found that starved rats were more sensitive than hypophysectomized rats, whereas Garcia and Van Dyke(3) found hypophysectomized rats and starved rats of equal sensitivity. In the present study meas-

urement of red cell radio-iron incorporation indicated that erythropoietic responsiveness of acutely starved rats was essentially the same as that of animals with erythropoiesis arrested by transfusion induced polycythemia (Fig. 4, Table II). The latter observation supports the thesis(11) that diminished erythropoiesis in the acutely starved rat is due to the relative plethora associated with starvation and hemoconcentration and not to the effect of food deprivation on the rat's ability to synthesize erythropoietin.

Comparison of dose-response relationship

for reticulocyte levels and red cell Fe^{59} incorporation showed that both increased as the dose was augmented, but reticulocyte response gave more variable results especially at lower dose levels in the transfused rats. This variability, thought to result from the method of reticulocyte counting, restricts use of this parameter to assay of relatively high doses of erythropoietin. Although measurement of red cell iron incorporation provided a simple and reliable means of measuring both high and low doses of erythropoietin, the amount of blood required for determination of Fe^{59} incorporation at the dose of radio-iron employed precluded the use of a single animal for serial determinations.

The dose-response analyses in these experiments were similar to those of Garcia and Van Dyke(3) and Hodgson *et al.*(5) in that the relationship between the log of the dose of urine concentrate or plasma extract in milliliters and the erythropoietic response was linear. Schleuter *et al.*(12) found a sigmoid relationship between Fe^{59} incorporation in erythrocytes of starved rats and the log dose of purified erythropoietin obtained from anemic sheep plasma. Within the range tested

in the present study a sigmoid dose-response relationship was not noted.

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Automatic Screening of Normal and Abnormal Electrocardiograms by Means of a Digital Electronic Computer.* (26260)

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In a digital computer program for automatic analysis of electrocardiograms (ECG) a screening procedure for separation of normal and abnormal records appears an essential preliminary step. A more detailed analysis of pathological tracings may then follow. Since the ECG consists of several distinct wave forms (P, QRS and T waves) with different electrophysiological significance a single screening procedure should preferably encompass analysis of all electri-

cal processes which accompany each heart beat. The spatial ventricular gradient ($\text{SV}\hat{\text{G}}$) appeared as the most comprehensive single parameter known for this purpose.

Wilson *et al.*(1) developed the concept of $\text{V}\hat{\text{G}}$. It has been defined as the net electrical effect of the differences in time course of ventricular depolarization and repolarization. As predicted by simple theory, the time integral ($\hat{\text{A}}$) of depolarization potentials of a muscle should be equal to those of repolarization but with opposite sign. For the ECG, the sum of the time integrals of QRS and T should be

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zero. In most ECG leads from normal hearts, however, QRS and T have the same polarity and the sum of their time integrals has a finite value. From this Wilson *et al.*(1) concluded that in normal hearts a difference in the time course between depolarization and repolarization must exist. $\hat{V}\hat{G}$ is an indicator of this difference ($\hat{A}\hat{Q}\hat{R}\hat{S} + \hat{A}\hat{T} = \hat{V}\hat{G}$).

Clinical application of $\hat{V}\hat{G}$ has always been very limited because its determination by graphic means is very time-consuming. Furthermore, many investigators found a poor separation between normal and abnormal tracings when $\hat{V}\hat{G}$ was determined in frontal plane projections only, as commonly done. To determine accurately the spatial characteristics of $\hat{V}\hat{G}$ ($\hat{S}\hat{V}\hat{G}$), however, weighted ECG leads with a constant ratio between generated current strength and recorded potentials and with constant direction are required (2). Conventional bipolar and unipolar leads do not fulfill these requirements. The more recently developed corrected orthogonal lead systems(2) have been designed according to these needs. Schmitt's SVEC III leads (3) were used in the present study.

Material and methods. Corrected orthogonal electrocardiograms(3) were recorded on magnetic analog tape from 122 normal subjects and 144 randomly selected patients with ECG abnormalities. ECG preamplifiers together with FM (frequency modulation) record amplifiers of the tape recorder† gave a flat frequency response from 0.1 to 1,250 c.p.s. An automatic analog-to-digital data converter with a sampling rate of 1,000/sec. for each ECG lead was used to re-record the tracings on digital magnetic tape. The conversion method has been described previously (4). The digital format chosen was that of the IBM 704 computer which was used for the study. The base line for determination of time integrals was computed as a straight line between averages of 15 digits preceding the P-wave and following the T-wave. Time integrals were then obtained for the entire cardiac complex by subtraction of negative

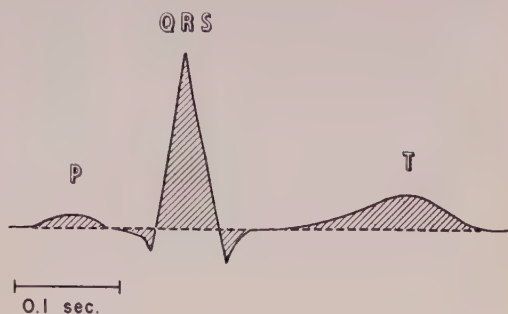


FIG. 1. Determination of ventricular gradient ($\hat{V}\hat{G}$) in one orthogonal lead. The time integral of total complex (P, QRS and T) is obtained by subtraction of negative areas from positive ones. The same procedure is performed in all 3 orthogonal leads. Results expressed in mVsec. Spatial ventricular gradient ($\hat{S}\hat{V}\hat{G}$) is then constructed as a spatial vector on the basis of the 3 time integrals obtained from the 3 orthogonal leads.

areas from positive ones (Fig. 1). The P-wave was included because the auricular gradient approaches zero and is, therefore, negligible.

Results. The following results were printed out by the computer: 1. $\hat{V}\hat{G}$ for each orthogonal lead; 2. Spatial magnitude of $\hat{S}\hat{V}\hat{G}$ derived from the orthogonal components, expressed in mVsec.; 3. Spatial direction of $\hat{S}\hat{V}\hat{G}$ in spherical coordinates (azimuth and elevation angles).

The mean magnitude of $\hat{S}\hat{V}\hat{G}$ in the group of 122 normals was 0.123 ± 0.047 mVsec. The mean direction was leftward, inferiorly and anteriorly (as related to the body of subjects). Only a few normal subjects showed a slightly posterior direction of $\hat{S}\hat{V}\hat{G}$. Two standard deviations of the mean results were used for determination of normal ranges for magnitude and spatial direction. Six cases fell out of these ranges.

Consequently, $\hat{S}\hat{V}\hat{G}$ of the 144 abnormal records was compared with the normal standards. Results are given in Table I. The overall accuracy of the decision "normal" or "abnormal" was 91% when compared with conventional clinical interpretations of the standard 12-lead ECG and vectorcardiograms of these patients. In the category of old myocardial infarctions only 1 case out of 56

† Ampex Corp., Type FR 104.

TABLE I.

	No. of cases	Abnormal in direction	Abnormal in amplitude	Total No. recog- nized as abnormal
		%	%	%
Myocardial infarction (old)	56	53 (95)	24 (43)	55 (98)
Left ventricular hypertrophy	34	24 (71)	14 (41)	28 (82)
Right " "	14	9 (64)	6 (42)	10 (71)
Left ventricular conduction defect	19	18 (95)	10 (53)	19 (100)
Right " "	21	16 (76)	12 (57)	19 (90)
Total	144	120 (83)	66 (46)	131 (91)

was not recognized as abnormal. The accuracy in recognition of cases with left or right ventricular hypertrophy was somewhat less, being 71 and 82% respectively. Directional discrepancies between normal and abnormal were considerably more significant than those of SV $\hat{G}$ magnitude (Table I).

Discussion. In the described procedure, ECG recording and data processing is trusted completely to technicians and automatic equipment. The physician needs to read and interpret, therefore, only those records which are recognized by the computer as being abnormal. This automatic procedure lends itself, therefore, to large-scale epidemiological studies of coronary artery disease and other cardiac disorders.

The accuracy of the screening process was determined by comparison of the results with conventional interpretations of the standard 12-lead ECG and vectorcardiograms (VCG). Burch *et al.*(5) and Hugenholtz *et al.*(6) have found in autopsy correlations that the diagnostic accuracy in recognition of myocardial infarcts is enhanced when VCG is used in addition to ECG. Since both were used in the present study, it appears that the diagnostic accuracy of the automatic screening procedure is at least as high as or higher

than that of the conventional 12-lead ECG alone.

Due to the small number of cases in some of the pathological subcategories, no attempt has been made to determine statistical ranges for these abnormal groups. Refinements of the described procedure may lead to grouping of cases according to specific ECG diagnoses.

Summary. The spatial ventricular gradient was determined in 256 electrocardiograms by means of a digital electronic computer. This automatic procedure allowed separation of normal and abnormal records with a high degree of precision. Of all cases with old myocardial infarctions, which are of prime interest in electrocardiographic diagnosis, 98% were recognized as abnormal.

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Antibody Response to Homografts VI. *In vitro* Cytotoxins Produced by Skin Homografts in Rabbits.* (26261)

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It is generally agreed that antibodies play a minor role in rejection of normal tissue homografts(1-4). Much of this conclusion is based on the failure of early attempts to demonstrate humoral cytotoxins following homografts of normal tissues(5-9). In contrast to these results, evidence suggesting the existence of cytotoxic antibodies to homografts has appeared in the past few years in various *in vivo* experiments(10-13). Moreover, direct evidence has been obtained of an *in vitro* toxicity of serum from animals injected with splenic cells(14-17). In view of these recent demonstrations of cytotoxins following grafts of homologous lymphoid tissues, the question of whether a simple skin homograft would elicit a similar antibody response was reexamined. The conditions under which these cytotoxins can be revealed and the time when they appear in the serum are reported.

Methods. Intra-breed or inter-breed skin homografts (4×6 cm) were applied on Dutch (D) or New Zealand (NZ) rabbits by the technic of Billingham and Medawar(18), and the recipient rabbits were bled at various intervals after grafting. Cytotoxicity of the serum was determined by incubating lymph node cells of the skin donor rabbit in serum dilutions of the skin graft recipient for 2 hours at 37°C , then examining the cells for viability by eosin dye exclusion as described by Hanks and Wallace(19). A phase contrast microscope with a $40\times$ objective was employed to count 200 cells for each determination. All tests were performed in a plastic tray containing $5/8 \times 5/16$ inch wells, using a moist blotter on the under surface of the cover to prevent evaporation. Cell suspensions were prepared by teasing out cells of the lymph node in Hank's balanced salt solution (B.S.S.) which contained 10%

normal rabbit serum, allowing clumps to settle, and adjusting the count to 50×10^6 lymphocytes per ml. To a total of 0.2 ml of normal or "immune" serum dilution in B.S.S., 500,000 lymphocytes were added per well. In most instances, fresh sera taken 2-6 hours prior to the test were used. For tests of sera stored at -10°C (sera No. 219, 220, 221, after first and second graft), following 1 hour incubation at 37°C , 0.2 ml of fresh normal rabbit serum was added to each dilution and incubation continued for 1 hour.

Results. Toxicity of sera from animals which have rejected skin homografts is summarized in Table I. Breakdown of all homografts was complete by the 20th day. Since the base level of viability of lymph node cells in normal serum varied somewhat from experiment to experiment (60-80%), a cytotoxic index (C.I.) was calculated as the difference between percentage of viable cells in experimental and control serum divided by the percent viable in control serum. Thus a maximal effect is shown by an index of 1.0 and a minimal effect by 0. Taking the viability in autologous serum as a control, the cytotoxic index of normal homologous sera and sera of autografted rabbits remained within narrow limits of -.17 to .09. It is evident that the index of sera from homografted rabbits was in most instances well above the control values. The mean cytotoxic index of the experimental sera diluted 1:2 was 0.30, as compared to control serum mean of -.01. The serum of rabbits 848 and 926 caused almost total destruction of donor lymph node cells during the 2-hour incubation period. If an index of 0.2 is selected as indicating a positive reduction in viable cells, the sera of 10 rabbits out of 18 can be considered to have contained toxic factors after a single graft. A 1:2 dilution of sera was more destructive than straight serum in a few samples, though the reason for this is

* This work was supported by grants from Nat. Inst. of Arthritis and Metab. Dis., U.S.P.H.S., and from the Calif. Inst. for Cancer Research.

TABLE I. Cytotoxic Effect of Serum from Normal and Homografted Rabbits on Lymph Node Cells of the Skin Graft Donor.

Donor of lymph node cells	Rabbit No.	Treatment	Serum tested	Time after grafting (days)	Cytotoxic Index (C.I.)* at serum dilutions:			
					1:1	1:2	1:4	
842 NZ	848 NZ	Homografted with skin once		25		1.0	.92	
" "	926 NZ			"		.92	.92	
" "	849 NZ			"		.07	.19	
752 D	840 NZ			21	.79	.17	.10	
" "	839 D			"		.17	.14	
" "	748 D			"		.08	.14	
959 NZ	962 NZ			23	.06	.25	.29	
" "	930 NZ			"		.43	.46	.35
" "	929 NZ			"		.03	.01	.03
960 NZ	955 NZ			38	.66	.68	.09	
" "	928 NZ			"		.24	.19	.15
" "	971 NZ			"		.09	.19	.02
740 NZ	226 D			11	.21	.44	.21	
" "	228 D			15	-.08	.08	.17	
218 NZ	221 D			19	.15	.11		
" "	220 D			"		.24	-.06	
" "	219 D			25	-.03	.02		
Mean of sera after first graft						.30		
Mean of 19 controls before homograft or autograft; or after autograft						-.01		
218 NZ	221 D	Homografted with skin twice		23	.47	.43		
" "	220 D			"		.77	.75	
" "	219 D			30	.63	.44		
" "	221 D	Homografted with skin 3 times		32	.67	.81	.59	
" "	220 D			"		.96	.38	.45
" "	219 D			"		.55	.34	.29
959 NZ	930 NZ			"	.84	.70	.78	
" "	929 NZ			"		.27	.25	.15
959 NZ	221 D	Homografted 3 times, tested with lymph node cells from a rabbit other than the skin donor		"	.37	.12	.29	
" "	220 D			"		-.01	0	
" "	219 D			"		-.03	-.10	
218 NZ	930 NZ			"	.03	.05	-.16	
" "	929 NZ			"		-.07	-.08	.05
960 NZ	221 D			"	.02	-.07	-.03	
" "	220 D			"		.55	-.07	-.17
" "	219 D			"		-.19	-.08	
" "	930 NZ			"	.31	.44	.47	
" "	929 NZ			"		-.02	-.11	

* Cytotoxic index = $\frac{C-E}{C}$, where C = % viable lymph node cells in control serum and E = % viable lymph node cells in experimental serum.

not immediately clear. Re-grafting for a second or third time can be seen significantly to increase the toxicity of serum. Also, the sera of 3 rabbits which were inactive following the first graft became toxic after a second or third graft (No. 219, 221, and 929). Some degree of non-specific toxicity was noted when the sera were tested with lymph node cells of rabbits other than the original skin donor (Table I). However, it is important

to note that the sera were always more deleterious to cells of the skin donor than to cells of any other rabbits. Moreover, non-specific toxicity was not found to the same degree against cells of 2 different individuals; thus the serum of rabbit 221 was slightly toxic to cells of rabbit 959, but not of rabbit 960, while the serum of rabbit 220 acted in the reverse fashion upon cells of the same 2 animals. Similarly, while the serum of rabbit

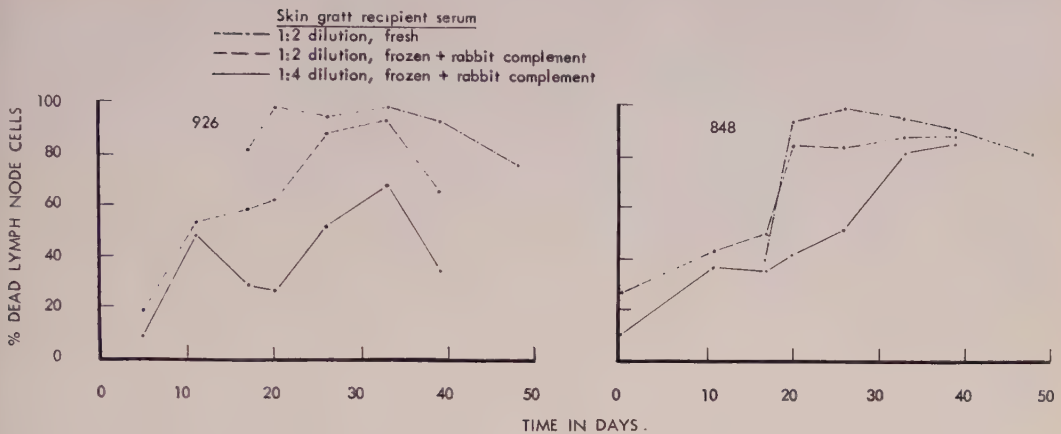


FIG. 1. Appearance of cytotoxins in sera of skin grafted rabbits to the skin donor lymph node cells.

930 was somewhat lethal to lymph node cells of No. 960, it was completely innocuous to cells of No. 218.

Time of appearance of lymphotoxins in the blood is illustrated in Fig. 1. Rejection of the graft had started in both rabbits by the 11th day and was complete by the 17th day in rabbit 926 and by the 20th day in rabbit 848. It is apparent that cytotoxins appeared in the serum when the graft began to show signs of degeneration, and reached a peak upon complete breakdown of the graft.

Discussion. One argument against the vital participation of antibodies in the homograft reaction to normal tissues is the inability to demonstrate any *in vitro* cytotoxicity of serum taken from a homografted animal. In the first extensive attempt, Medawar found that sera taken from rabbits homografted with skin did not affect survival, frequency of cell division, or migratory activity of tissue cultured skin cells from the donor animal(6). With similar tissue culture techniques, Allgower, Blocker and Engley were also unable to find any deleterious effect of "immune serum" on cultured skin cells(7). The difficulty that all cells of the explant may not have been exposed to the serum was overcome in later experiments by Billingham using dissociated epidermal cells(20). Although no evidence of direct cytotoxicity of immune serum was found, a "protective" effect was shown by retransplantation of the epidermal cells after *in vitro* incubation with

immune serum. It was thought that the application of immune serum to the Malpighian cells *in vitro* may have caused them to provoke and succumb to nonspecific vascular inflammatory reactions on subsequent transplantation.

In the present experiments, it has been possible to demonstrate *in vitro* cytotoxins following a simple skin homograft by using lymph node cells as the target cell. Cytotoxicity was quite marked in most instances; the sera of 2 animals killed almost all the lymph node cells of the skin donor within 2 hours. Though the sera of some animals were not toxic, an average 30% depression in viability of cells was produced by the sera of 18 rabbits. The following evidence indicates that this toxicity is probably caused by factors having general characteristics of antibodies and not by non-specific toxins. 1). Normal homologous sera were innocuous. Following the application of skin homografts, peak activity was reached in 20-30 days. 2). Cytotoxicity of the sera increased following a second or third skin graft from the original donor. 3). Heating sera at 56°C for 30 min abolished its activity and addition of fresh normal rabbit serum restored toxicity (unpublished). 4). To a large extent, toxicity was specifically directed against the cells of the skin donor rabbit. Some cross reactivity occurred with cells of some non-specific rabbits, but not with others. This could be the result of

sharing of common antigens by the cells tested.

Although it was shown that lymphoid cells are susceptible to serum factors of a skin-grafted rabbit, it remains to be seen whether other types of cells are also affected. Preliminary tests have shown that polymorphonuclear leucocytes of the skin donor are agglutinated by active sera and that epidermal cells are killed by such sera.

Summary. The sera of rabbits which were homografted with skin are shown to be toxic to lymph node cells of the skin donor rabbit. Cytotoxicity was demonstrated upon incubation *in vitro* for 2 hours at 37°C. Sera of grafted rabbits became toxic after graft breakdown had commenced (11 days), and reached a peak after the rejection (20-30 days). The sera were in all instances more toxic to lymph node cells of the skin donor than to cells of other rabbits.

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Negative Feedback Mechanism of Cholesterol Synthesis in Experimental Nephrosis.* (26262)

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(Introduced by A. B. Eisenstein)

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To date the mechanism for production of hypercholesterolemia in the nephrotic syndrome is unknown. Various postulates have been offered including hypothyroidism(1), increased intestinal cholesterol absorption

(2), defective lipid transport(3), etc. Recently the existence of a negative feedback mechanism for hepatic cholesterol synthesis has been demonstrated in normal animals (4,5). It seemed possible that an absence of, or a defect in this mechanism might explain, at least in part, the increase in serum cholesterol in nephrosis. To test this possibility, the effects of cholesterol feeding upon normal and nephrotic animals were studied. Observations were made of C¹⁴-acetate incorpora-

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TABLE I. Effect of Cholesterol Feeding on Liver Slice Incorporation of C^{14} Acetate into Cholesterol.

	# rats	Proteinuria, mg/24 hr	Diet	C^{14} , cpm/mg liver Cholesterol-digi- tonide	P value (# test)
Pair-fed normal	6	0	Normal	$994.9 \pm 324.2^*$	} <.025
	6	0	2% cholesterol†	81.1 ± 14.5	
Pair-fed nephrotic	9	214.0†	Normal	535.9 ± 141.8	} <.001
	10	214.5	2% cholesterol	45.7 ± 38.4	

* Mean $\pm$ stand. error of mean.

† 5 day diet.

‡ Mean.

tion in cholesterol in hepatic tissue slices and of the disposition of cholesterol in various tissues.

The results indicate that in experimental nephrosis, the hepatic feedback mechanism is operational and normally responsive to exogenous cholesterol. Certain abnormalities of cholesterol disposition were noted.

Material and methods. Sixty-four male rats of the Sprague-Dawley strain with starting weights of 100-120 g were studied. Animals were housed individually and pair-fed cholesterol-containing and control diets for periods of 5 to 15 days prior to sacrifice. The cholesterol diet was prepared by addition of 2% cholesterol U.S.P. to ground Purina chow, after solution of cholesterol in ether, while the control diet was prepared accordingly with the solvent alone.

The nephrotic syndrome was produced in 35 animals by 11 daily subcutaneous injections of the aminonucleoside of puromycin as previously described(6,7). The classical picture of nephrosis with proteinuria, ascites and hypercholesterolemia was evident by the tenth day. Urinary protein was estimated by the Esbach method(8).

The experiments were divided into 2 types. In the first, studies were designed to measure the effect of cholesterol feeding on C^{14} -acetate incorporation into liver slice cholesterol. Normal rats fed a 2% cholesterol diet were pair-fed with normal animals on the control diet. Nephrotic rats were similarly pair-fed against each other.

Modifications were made of the Baruch and Chaikoff(9) method for assaying incorporation of acetate 2- C^{14} into cholesterol in hepatic tissue slices. Slices weighing 500 ± 5 mg with a thickness of about .5 mm were

cut with the aid of a plastic hand-microtome. Incubations in duplicate for each animal were carried out in flasks containing 4.5 ml of Krebs-Ringer phosphate buffer, 0.5 ml of cold sodium acetate (2.49 mg) made isotonic with NaCl and tracer amounts of sodium 2- C^{14} acetate (specific activity 9075 cpm/ μ M). Flasks were gassed with 95% oxygen and 5% CO_2 for 3 hours at 37°C in a Dubnoff shaker. After hydrolysis, saponification and extraction of the non-saponifiable lipids, cholesterol was precipitated in chloroform with a .5% solution of digitonin (Sperry method); the precipitate was filtered (Whatman #50 paper cut to fit the planchettes) and after washing and drying, counted in a proportional gas flow counter at infinite thinness.

The second type of experiment was designed to study the distribution of cholesterol in tissues of normal and nephrotic rats before and after cholesterol feeding. In these experiments, normal rats were pair-fed against nephrotic rats both on control diets and cholesterol-containing diets. Cholesterol determinations in serum and tissue homogenates were carried out by the method of Saifer and Kammerer(10). All animals were sacrificed by decapitation after an overnight fast except for animals used for the C^{14} -acetate experiments, which were fed until time of sacrifice, to prevent the starvation effect upon acetate incorporation.

Results. Hepatic cholesterol feed-back: In Table I it may be seen that C^{14} -acetate incorporation into cholesterol by liver slices of nephrotic animals on a normal diet is considerably less than that in normal animals (54% decrease). It is likely that this difference reflects the fact that food intake of nephrotic animals compared with normals is

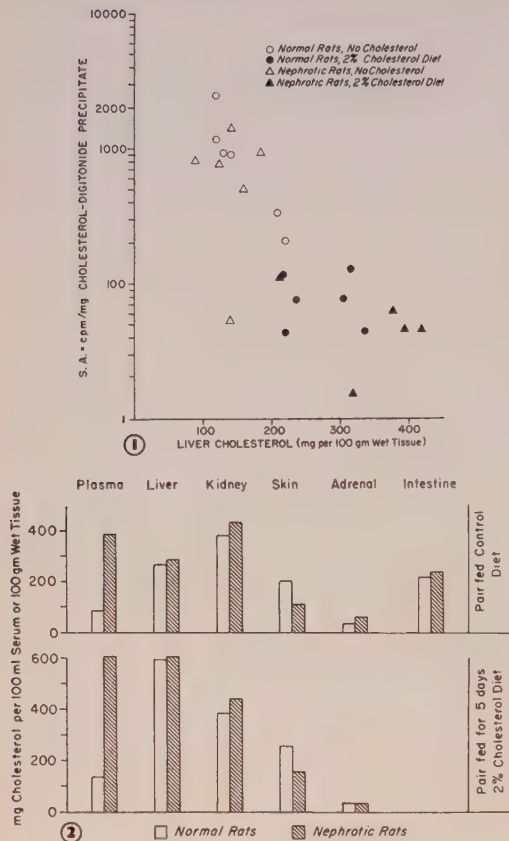


FIG. 1. Relationship between S.A. liver cholesterol and concentration of liver cholesterol in normal and nephrotic animals before and after cholesterol feeding. Of the 16 nephrotic rats noted in Table I, in only 11 animals were studies made of liver cholesterol concentration as well as S.A. of liver cholesterol.

FIG. 2. Effect of cholesterol feeding on distribution of cholesterol in tissues. Sixteen rats were used in each group of pair-feeding experiments.

decreased. After 5 days of cholesterol feeding, both groups of animals show almost complete inhibition of hepatic cholesterol synthesis (91.9% reduction in controls and 91.5% in nephrotics). Since the level of hepatic cholesterol doubled (Fig. 1) in both groups of animals after cholesterol feeding, it seems reasonable that a simple dilution effect was not completely responsible for the striking depression in specific activity of liver cholesterol. Fig. 1 also shows the relationship between specific activity of the cholesterol-digitonide precipitate and cholesterol content of liver. Despite considerable scatter, it may be noted that an inverse relation-

ship exists between the two. These findings suggest that a negative feed-back mechanism for hepatic cholesterol synthesis obtained in the nephrotic animal, is dependent upon liver cholesterol concentration, and does not differ from the normal.

Tissue distribution of cholesterol. Distribution of tissue cholesterol may be seen to be quite similar in liver, kidney, adrenal, and intestine (Fig. 2) of pair-fed nephrotic and normal rats maintained on a normal diet. However, skin and plasma levels differ. Feeding of a 2% cholesterol diet results in a profound increase in hepatic cholesterol in both groups. The magnitude of this change appears similar, suggesting that nephrotic liver is capable of trapping cholesterol. Other tissues behave similarly in both groups of rats with kidney and adrenal showing no change, and skin increasing somewhat. Plasma response differs, in that the absolute rise in nephrotics from their control levels is considerably greater (200 mg%) than the rise in plasma levels in normal rats (30 mg%).

These observations indicate that nephrotic tissues, excluding plasma, behave as do normal tissues in response to exogenous loads of cholesterol.

Discussion. Gould and Taylor(11,12) and Frantz, *et al.*(13), working with hepatic tissue slices of normal animals, have shown that addition of cholesterol to the diet causes a depression in rate of synthesis of cholesterol in the liver. Studies on incorporation of C^{14} -acetate into cholesterol in liver slices obtained from rats made nephrotic with anti-kidney serum have been reported by Heyman (14) and Marsh and Drabkin(3). The former investigator found no change in cholesterol synthesis in 9 animals sacrificed within 2 days after injection of the nephrotoxic serum. In contrast, the latter authors reported(15) an increased *in vitro* and *in vivo* hepatic cholesterol synthesis 48 hours after nephrosis was established. Byers, *et al.*(16) found no change or only a slight decrease of hepatic cholesterol formation in nephrotoxic-nephrotic rats. Marsh and Drabkin(3) obtained decreased hepatic cholesterol formation and unchanged renal cholesterol synthe-

sis in rats 5 to 7 days after induction of the nephrotic state.

Our data obtained in severely and acutely nephrotic rats, in agreement with the work of Marsh and Drabkin, demonstrate a statistically significant decrease in hepatic cholesterol synthesis ($P < .01$) in nephrotic animals, although these animals were not paired with normals.

It may be possible to resolve some of these differences in terms of the nutritional state of the animals studied. Since nephrotic animals may be partially starved as the disease progresses, it is likely that decreased synthesis may be noted in later stages of the disease. In general, it would appear from our observations that hepatic cholesterol synthesis is certainly not increased.

Cholesterol added to the diet for 5 days decreased the rate of hepatic cholesterol synthesis to very low levels in both normal animals ($P < .025$), and nephrotics ($P < .001$). Feeding this diet for 15 days in 6 nephrotic animals did not further decrease hepatic cholesterol synthesis. It does not seem, therefore, that a faulty feedback mechanism can be invoked to explain nephrotic hypercholesterolemia.

The response of nephrotic animals to exogenous cholesterol appeared to be similar to normal except as to retention of cholesterol in plasma. Assuming a plasma volume of 6 ml per rat, normal rat plasma retained 1.8 mg of cholesterol while nephrotics retained 12 mg of cholesterol (Fig. 2). This type of difference could be explained by alteration in plasma binding of cholesterol in nephrosis. It seems unlikely that the problem is a tissue (hepatic) inability to take up cholesterol, since nephrotic liver and other tissues responded as did normal tissue to exogenous cholesterol. On the other hand, since normal liver cholesterol doubled with only a small increase in plasma cholesterol level, one might have anticipated a considerably greater

increase in nephrotic liver cholesterol relative to the plasma increase.

These findings do not permit differentiation between a hepatic defect or a plasma defect but do suggest an abnormality of the interrelationship.

Summary. Feeding of cholesterol resulted in marked inhibition of hepatic cholesterol synthesis in nephrotic and normal animals. A faulty hepatic feedback mechanism cannot be invoked to explain nephrotic hypercholesterolemia.

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Inactivation of Prolactin by Human Blood Serum and Plasmin*† (Fibrinolysin). (26263)

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Inactivation of several protein hormones by various biological tissues has been reported. Adrenocorticotropin (ACTH) is readily inactivated when incubated with whole blood, blood plasma(1-3); or fibrinolysin(4); glucagon, ACTH and somatotropin are inactivated by streptokinase-activated blood plasma or plasminogen(5,6); and insulin, ACTH and prolactin activities are reduced by various mammalian tissues and tissue homogenates (7-10). The present report deals with the effects on prolactin activity of incubation with human blood serum and fibrinolysin (derived from human blood).

Methods. Purified ovine prolactin‡ with an activity of 15 I. U. per mg was used in these studies. The prolactin was dissolved in distilled water to a concentration of 5.0 mg prolactin per ml solution; 0.1 ml (0.5 mg) prolactin solution was added to each flask which contained 0.9 ml incubation medium. Serum concentrations were prepared by diluting pooled serum, representing 6 different blood samples, with Krebs-Ringer phosphate buffer of pH 7.2. Three consecutive assays were performed employing pooled serum which was aliquoted, stored in a freezer and thawed just before use. Plasmin§ solutions were prepared by adding 0.1 ml (5.7 caseinolytic units; 14.1 Loomis units) fibrinolysin solution to 0.8 ml Krebs-Ringer phosphate buffer. Total volume of each incubation sample was 1.0 ml. As a control, standard prolactin was added to Krebs-Ringer phosphate buffer and incubated simultaneously with the serum and fibrinolysin preparations. Following 2 hours incubation at 37°C, each sample was diluted 10-

fold with saline solution to give an initial prolactin concentration of 0.05 mg/ml.

Prolactin activity was assayed in White Carneau pigeons employing the method of Reece and Turner(11). Each pigeon was given 0.1 ml assay solution daily for 4 days. On the fifth day the pigeons were sacrificed and the crop sacs were removed and rated. By injecting the incubated standard prolactin preparation over one crop sac and comparing the response with that of an equivalent amount of experimental sample injected over the other crop sac, each bird served as its own control. To minimize subjectivity, ratings were made by 2 independent co-workers who were unfamiliar with the assay materials but experienced with the assay technic.

Results. Incubation of prolactin with human blood serum and with fibrinolysin caused significant losses in prolactin activity (Tables I and II). Incubation of 0.5 mg prolactin in 10% serum (Table I) resulted in moderate but significant losses (33, 36 and 40%) of prolactin activity. The activity of a similar amount of prolactin incubated in 45% serum produced a mean pigeon crop re-

TABLE I. Mean Loss of Prolactin Activity Following Incubation in Human Blood Serum. (0.5 mg prolactin incubated in each exp.)

No. pigeons used per assay	Avg rating of pigeon crops		Loss of prolactin activity	
	Std. prolactin	Incubated in 10% serum	mg	%
4	2.56	1.50	.20	40
4	1.56	1.00	.18	36
4	1.40	.95	.17	33
		Avg	.18	36 ±2.0*
		Incubated in 45% serum		
3	1.91	.58	.35	70
5	1.70	1.00	.21	41
3	2.42	1.42	.26	52
		Avg	.27	54 ±8.5*

* Stand. error.

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‡ Supplied by Endocrinology Study Section, N.I.H.

§ We wish to thank The American National Red Cross and Dr. J. Sgouris of Mich. State Health Labs for the fibrinolysin solution.

TABLE II. Mean Loss of Prolactin Activity Following Incubation with Fibrinolysin (5.7 eu). (0.5 mg prolactin incubated in each exp.)

No. pigeons used per assay	Avg rating of pigeon crops		Loss of prolactin activity	
	Std. prolactin	Incub. with fibrinolysin	mg	%
7	1.39	.21	.43	85
7	1.57	.07	.48	96
10	1.37	.0	.50	100
		Avg	.47	94±5.2*

* Stand. error.

sponse that was only 46% of the standard response, indicating a 54% loss of prolactin activity. Incubation with fibrinolysin resulted in essentially a complete loss (85, 96 and 100%) of prolactin activity (Table II).

Discussion. These data show that human blood serum and human fibrinolysin can inactivate ovine prolactin. The factor(s) in human blood serum which inactivate prolactin is unknown; however, it is possible that the fibrinolysin system is at least partly responsible for the loss of activity. Previously it was demonstrated that prolactin was inactivated by several other tissues, including mammary gland, ovaries, liver and kidney of rats and pigeon crop glands(9,10). Similarly it has been shown that ACTH is inactivated when incubated with rat liver and kidney slices, diaphragm, and homogenates of liver, kidney and adrenal tissues(8). The inactivating systems of these tissues were not described.

Fibrinolysin is a proteolytic enzyme, found in all mammalian blood sera, but normally present as the inactive precursor, plasminogen(12). Addition to blood plasma of factors known to activate plasminogen has increased the hormone-inactivating efficiency of human plasma(6). Recently it has been shown that a "factor" extracted from human blood plasma will activate (a) plasminogen and (b) blood plasma fractions in which plasminogen is concentrated (Hopkins, Turner and Pincus, unpublished). Both activated

products readily inactivated ACTH.

Since prolactin was inactivated by human blood serum (and more recently by bovine, rabbit and rat sera, unpublished) without addition of known plasminogen activators, we investigated the presence in prolactin of plasminogen-activator contaminants. In the fibrin plate test, prolactin activated human plasminogen, causing lysis of fibrin. The presence of plasminogen activators has previously been demonstrated in human pituitary tissue(12). Inactivation of prolactin by human serum and activation of plasminogen by prolactin cannot be interpreted at this time to indicate that the fibrinolysin system alone is responsible for loss of prolactin activity.

Summary. Ovine prolactin (7.5 I.U.) was incubated for 2 hours at 37°C with human blood serum and human fibrinolysin. Moderate decreases of prolactin activity occurred after incubation in 10% serum, and somewhat greater losses occurred when incubated in 45% serum. Fibrinolysin caused almost complete inactivation of prolactin. The relationship of the serum inactivator system to the fibrinolysin system remains to be established.

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Functional Response of Regenerating Adrenal Glands to Stress.* (26264)

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Although enucleation of the adrenal gland in the rat is followed by rapid regeneration, the mass of regenerated cortical tissue never comes to equal that of both glands in normal animals of the same size, age and sex(1). Previous reports have shown that regenerating adrenal cortical tissue can respond to nonspecific stress(1) but, whether or not normal functional capacity is ever regained by such glands has not been clearly established. Development of micro-methods for determination of corticosterone in plasma has made possible the *in vivo* study of adrenal cortical functional capacity under many experimental conditions. Guillemin *et al.*(2) have shown, for example, that the level of systemic plasma corticosterone accurately reflects the secretory activity of the adrenal cortex. Utilizing such methods, Fortier and De Groot(3) showed that rats bearing a single regenerating adrenal had a normal resting plasma corticosterone level as early as 8-16 days after enucleation. The object of the present study was to determine *in vivo* the functional response of regenerating adrenal glands to nonspecific stress throughout the period of adrenal regeneration as reflected by the corticosterone level of systemic plasma.

Methods. Three groups each consisting of 128 female Holtzman rats weighing 50-60 g were (A) unoperated, (B) bilaterally adrenal enucleated, and (C) unilaterally adrenalectomized and unilaterally adrenal enucleated, respectively. At 2, 3, 6 and 12 weeks, 24 animals from groups A, B, and C were stressed by bilateral fracture of the tibia and fibula under ether anesthesia. Thirty, 60 and 120 minutes after stress the rats were decapitated, blood was collected from the severed neck in heparinized beakers and plasma obtained as rapidly as possible following centrifugation in the cold. Plasma from non-

stressed animals was obtained from 8 rats of groups A, B and C after 12 hours isolation in a dark, quiet environment. Adrenals were removed, freed of adherent fat and weighed to nearest tenth of a milligram.

Corticosterone was determined in plasma by the fluorometric method of Zenker and Bernstein(4). In agreement with others, (2,3) a relatively constant residual fluorescence was found in the plasma of rats 48 hours after bilateral adrenalectomy which cannot be ascribed to corticosterone. Aliquots of plasma pools from stressed rats were analyzed for corticosterone by this fluorometric procedure and by an isotopic dilution technic using corticosterone-4-C¹⁴† similar to that described by Peterson(5). Corticosterone accounted for 90% of the fluorescence in plasma from stressed rats after the value for constant residual fluorescence had been subtracted from total fluorescence determined by the Zenker-Bernstein method. This agrees well with previously published data on the validity of other fluorometric procedures for corticosterone determination in rat plasma (2).

Results. Results are illustrated in Fig. 1-4. In all instances plasma corticosterone of control rats with intact adrenals increased approximately 8 to 10 fold following initiation of stress. At 2 weeks plasma corticosterone of animals bearing a single regenerating gland increased less than 2 fold and those bearing 2 regenerating glands increased less than 4 fold in response to stress. At 3, 6 and 12 weeks a progressively greater rise of plasma corticosterone occurred in response to stress in rats with one or 2 regenerating adrenals, but in neither circumstance was a normal response attained. The rise in plasma corticosterone following stress was greater in animals with 2 than with 1 regen-

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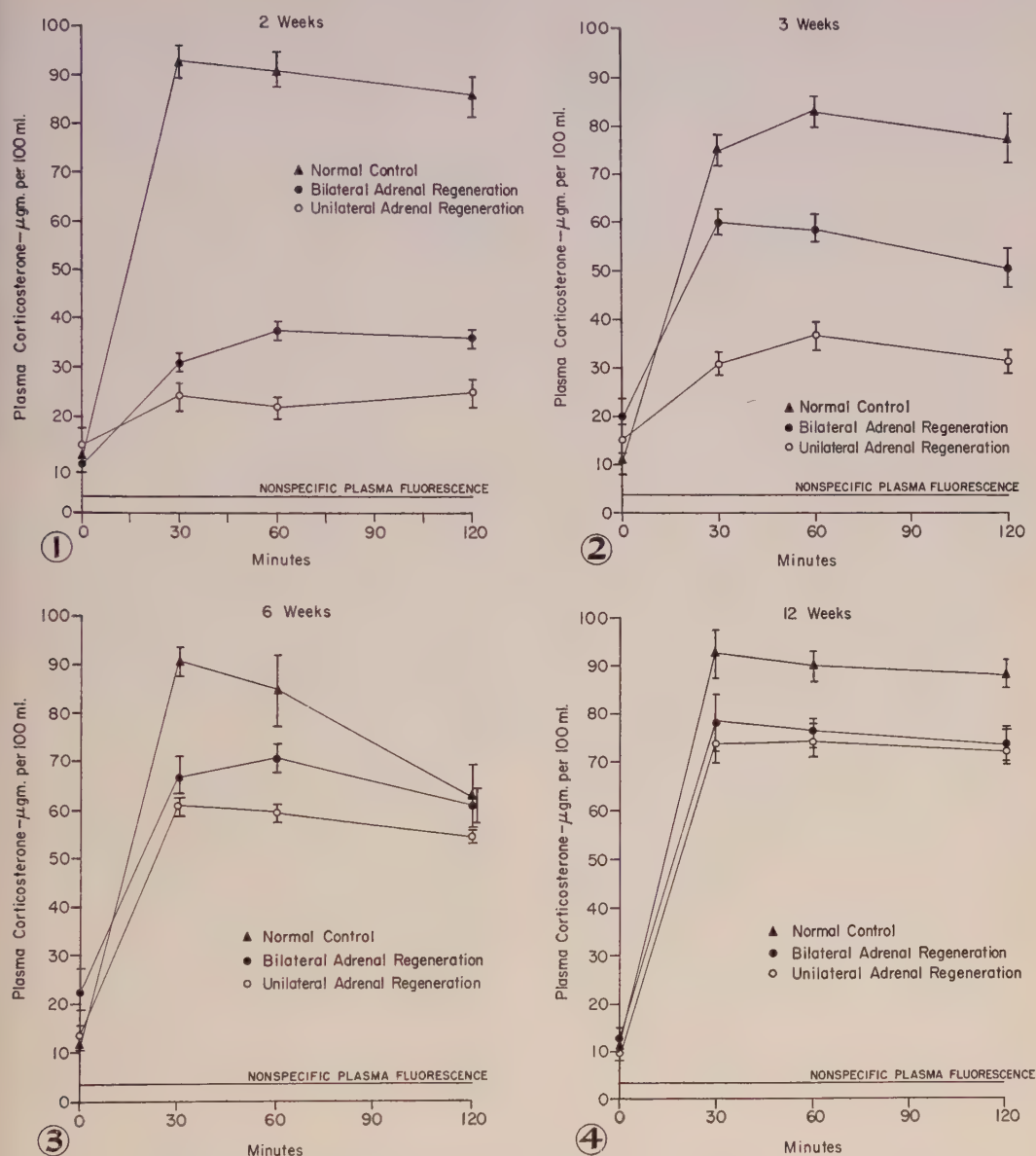


FIG. 1-4. Response of normal and regenerated adrenal cortex to stress as reflected by changes in plasma corticosterone levels. Vertical bars represent stand. error of the mean.

erating gland at 2, 3 and 6 weeks, but at 12 weeks no difference was apparent. When calculated as a percentage of the response in control animals (Table I), rats with 2 regenerating glands showed a more rapid return toward normal than those with one regenerating gland. Maximum response occurred after 12 weeks regeneration when the mean 2 hour response of rats with 2 regenerated

glands and one regenerated gland was 84% and 82% of control values, respectively (Table I). Calculation of regenerated cortical mass according to data reported by De Groot and Fortier(3) (Table I) shows that the magnitude of plasma corticosterone increase following stress was greater than the increase in weight of the regenerated cortex, suggesting that response was not solely de-

TABLE I. Response of Normal and Regenerated Adrenals to Stress as Reflected by Plasma Corticosterone Levels.

Weeks	Plasma corticosterone, $\mu\text{g}/100\text{ ml}$, mean 2 hr response*			% response		
	Control	Bilat. regen.	Unilat. regen.	Bilat.: Control	Unilat.: Control	Unilat.: Bilat.
2	89.8 $\pm$ 2.3†	34.8 $\pm$ 1.6	23.4 $\pm$ 1.6	39	26	67
3	77.6 $\pm$ 5.1	56.7 $\pm$ 2.1	32.9 $\pm$ 1.5	73	42	58
6	79.4 $\pm$ 4.1	66.1 $\pm$ 2.3	58.7 $\pm$ 1.2	83	74	89
12	90.1 $\pm$ 2.3	76.4 $\pm$ 2.5	73.8 $\pm$ 2.2	84	82	97
Weeks	Calculated cortical wt, mg			% ratio of calculated cortical wt		
	Control	Bilat. regen.	Unilat. regen.	Bilat.: Control	Unilat.: Control	Unilat.: Bilat.
2	43.9 $\pm$.7	18.2 $\pm$.5	11.9 $\pm$.5	41	27	65
3	38.0 $\pm$.7	22.4 $\pm$.5	15.8 $\pm$.4	59	42	70
6	41.9 $\pm$.7	27.8 $\pm$.6	21.7 $\pm$.5	66	52	78
12	44.4 $\pm$.8	34.6 $\pm$.6	25.0 $\pm$.7	78	56	72

* Avg of uncorrected values obtained at 30, 60 and 120 min. after stress.

† Stand. error of mean.

pendent upon mass of regenerated adrenal cortical tissue.

Discussion. Decrease in adrenal concentration of ascorbic acid, lymphoid atrophy, fall in circulating eosinophils and survival in the face of exposure to various noxious stimuli has indicated that regenerated adrenals were capable of functional response to non-specific stress, but whether or not a normal response in terms of hormone production was ever attained has remained in doubt. *In vitro* incubation studies of Masson, Koritz and Peron(6) demonstrated that regenerating adrenals (21 and 30 days after enucleation) secreted corticosterone into the incubation medium but the amount secreted by regenerated adrenal tissue was less than that of normal adrenal tissue, especially when ACTH was added. Corticosterone secretion into adrenal vein blood also has been reported to be less from regenerating glands than from intact adrenals(6). Although these observations probably reflect decreased functional response of such glands to ACTH, both methods have made it difficult to quantitate the magnitude of the functional response of regenerating adrenal tissue to stress since *in vitro* procedures are unphysiologic and adrenal vein cannulation is itself a stress. The experiments reported here obviated these difficulties inasmuch as corticosterone levels were determined in systemic blood plasma under physiologic conditions of stress and non-stress. Results confirm the marked functional deficiency of regenerating adrenal

tissue at 2 weeks and, although functional capacity increased with time such glands failed to regain the ability of normal adrenals to elevate systemic blood levels of corticosterone in response to stress. The greater increase in functional capacity than in mass of regenerated tissue also lends support to the suggestion that regenerated tissue is more active per unit of weight than normal adrenal cortical tissue.

Summary. Plasma corticosterone changes were studied in response to stress in normal rats and in animals bearing 2 regenerating adrenals and 1 regenerating adrenal at intervals of 2, 3, 6 and 12 weeks after enucleation. Such glands were capable of elevating systemic blood corticosterone levels in response to stress by 2 weeks, the increase being greater in those with 2 regenerating glands than in those with one. The functional capacity of regenerating adrenals as determined by the elevation in plasma corticosterone following stress progressively increased from minimal ability at 2 weeks to maximum capability at 12 weeks, but the functional capacity of normal adrenals was never regained.

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Connective Tissue. II. Distant Dermal Collagen Response to Local Inflammation.* (26265)

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It has been demonstrated that there is a significant decrease in total dermal concentration of hydroxyproline or collagen in skin distant from the site of local croton oil induced inflammation(1,2,3). This loss of dermal collagen from uninjured tissue is not associated with a change in either total dermal nitrogen or hexosamine(3).

Recently, we have developed a technic for extractive dissection of rat skin into ground substance, neutral soluble collagen, procollagen and insoluble collagen(4,5). Employing this procedure, we have investigated the nature of the "distant dermal collagen response to local inflammation."

Material and methods. Twenty-four Sprague-Dawley rats, averaging 270 g in weight were divided into 3 equal groups of 8 each, 2 of which were injected intradermally with 0.4 ml of a 75% solution of croton oil in peanut oil, the third group with a similar volume of the carrier oil alone. This latter group was sacrificed by etherization within a few minutes after injection, and 5 g of shaved abdominal skin distant from site of injection (left abdominal area) were removed. The chemical analysis of skin from animals injected with carrier alone did not differ significantly from normal 0, 4 or 14 days after injection(3). The eschar formed 4 days after injection of the irritant, and one group of animals was sacrificed at this time. Similarly, the third group was killed after 14 days when the eschar had sloughed, and the apparently uninjured skin collected. All tissues were shaven, dissected clean of adhering fat, fascia and muscle, and finely minced.

Triplicate samples of pooled tissue from 8 animals were extracted sequentially in 0.15 and 0.5M NaCl and citrate buffer as described previously(5). The hexosamine(6), hydroxyproline(7) and nitrogen content of the extracts, as well as that of the whole skin of each animal separately, was determined in triplicate after acid hydrolysis(5). These extracts have been shown to remove quantitatively the ground substance, neutral soluble collagen and "procollagen" respectively(5). The difference between total tissue content of each component and the sum of the soluble material in μ moles/g of starting tissue defined the composition of the insoluble residue.

All results were expressed in μ moles/g of skin as the mean of triplicate analyses performed upon triplicate tissue extracts obtained from the pooled tissue of each group of 8 animals. The analytical variation between the triplicate extracts obtained from each group of animals was less than 10%. Mean values of 9 determinations for each fraction are presented along with the appropriate standard deviation below. The tissue samples were stored for a few weeks in the frozen state after cleaning but prior to either hydrolysis or extraction. Water loss *via* sublimation was essentially constant for all tissues at 30% w/w as has been described previously(4).

Results and discussion. The pathology of this type of injury, a non-suppurative ulcerous defect lightly outlined by erythema, has been described(2). No significant changes in dermal hexosamine or nitrogen were found in the skin distant from site of local croton oil induced inflammation. Changes in hy-

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TABLE I. Distribution of Dermal Hydroxyproline (μ moles/g) in Rat Skin Distant from Site of Local Croton Oil Induced Injury.

Extract	Days after injury		
	0	4	14
.15M NaCl	7 $\pm$.4	7 $\pm$.6	8 $\pm$ 1
.50M "	29 $\pm$ 2	* 15 $\pm$.8	* 12 $\pm$.6
.50M citrate buffer	12 $\pm$.6	* 43 $\pm$ 3	* 52 $\pm$ 3
Insoluble	312 $\pm$ 20	* 185 $\pm$ 14	* 224 $\pm$ 13
Total	360 $\pm$ 15	* 253 $\pm$ 15	* 296 $\pm$ 16

* Significantly different ($p < .05$) from control.

droxyproline were significant, however (Table I). These data demonstrate that while there was no change in isotonic saline soluble hydroxyproline, there was a profound drop in dermal concentration of neutral soluble collagen and insoluble collagen. These decreases occurred in the face of a marked increase in procollagen. These dermal collagen changes were not significantly affected by either formation or sloughing of the eschar.

Dermal extracts were mixed 5:1 with 40% trichloroacetic acid. Over 95% of the soluble nitrogen was precipitated by this procedure. Therefore dermal protein content of skin is accurately measured by total nitrogen, the constancy of which indicates that insoluble collagen is specifically decreased in skin distant from the site of local injury, and that there is no general protein catabolic response to inflammation.

The specificity of this dermal collagen response to local inflammation suggests the possibility that a dermal collagenolytic enzyme is hydrolysing the insoluble collagen. Recently a mammalian collagenase has been described(8). Quantitatively, the increased concentration of procollagen cannot account for the loss of insoluble protein. More probably, collagenolysis would result in peptide sized products, which would be soluble in either saline fraction. Since no increase in saline soluble hydroxyproline was found, these materials may drain away from the skin *via* dermal lymphatics.

Rat skin from animals subjected to massive daily doses of sodium dialantin also contain less neutral soluble collagen and more procollagen than normal skin(4). These der-

mal collagen changes with dilantin result in an increased synthesis of insoluble collagen, however. If such changes in soluble dermal collagen are characteristic of the synthesis of insoluble collagen, why with inflammation are similar changes in soluble collagens found associated with a profound decrease in insoluble and total dermal collagen? Perhaps the distant dermal collagen response to local inflammation involves firstly the enzymatic hydrolysis of insoluble collagen and secondly in response to this loss, an attempted synthesis of replacement collagen. Rate of insoluble collagen synthesis being slower than rate of hydrolysis(4,9), a steady state would be established in which dermal concentration of 0.5M saline soluble collagen was low, since it rapidly aggregates to form procollagen(4), and this latter component would increase in concentration because of the slower rate required for collagen fiber formation(9).

Collagen concentration in mg/g of skin is equal to the concentration of hydroxyproline in μ mole/g and total non-collagenous protein may be calculated from the difference between total and collagenous nitrogen as previously described(5). After correction for dermal water loss (30%) *via* sublimation with storage of tissue samples in the frozen state(5), the chemical anatomy of the skin before injury, after eschar formation and after eschar sloughing is shown in Table II. Water content was determined by lyophilization and fat by difference. This table clearly demonstrates the specific decrease in dermal collagen with local inflammation, and accumulation of non-collagenous protein.

Summary and conclusions. Skin distant from local croton oil induced inflammation demonstrates no significant change in total hexosamine or nitrogen, despite a marked de-

TABLE II. Chemical Anatomy of Rat Skin Distant from Site of Local Injury.

Component (mg/g)	Days after injury		
	0	4	14
Collagen	240 $\pm$ 10	* 169 $\pm$ 10	* 200 $\pm$ 10
Other protein	150 $\pm$ 8	* 230 $\pm$ 12	* 200 $\pm$ 10
Water	550	550	550
Fat	60	51	50

* Significantly different from control.

crease in 0.5 M saline soluble, insoluble and total collagen. Since less than 5% of total soluble nitrogen is not protein, this constancy of dermal nitrogen means that non-collagenous dermal proteins or glycoproteins are being synthesized. Therefore insoluble collagen is specifically decreased in response to local inflammation. It is suggested that this distant dermal collagen response to local inflammation results from enzymatic collagenolysis which stimulates the attempted synthesis of replacement collagen. A steady state with respect to collagen anabolism and catabolism could then be established in which, by reason of the relative rates of synthesis and degradation, the skin would contain subnormal amounts of 0.5M saline soluble and insoluble collagen and supernormal

concentrations of pro-collagen.

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Placental Transfer of Fluorine in the Human Fetus.* (26266)

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Recent studies have established certain relationships between daily fluorine intake by pregnant women and fluorine content of the maternal blood, placental tissue and cord blood(1,2,3,4). Gardner *et al.*(1) found significantly higher fluorine values in maternal blood and placental tissues of pregnant women living in Newburgh, N. Y. where fluorine content of the water is 1 ppm, than in those living in Rochester, N. Y., where the water is fluorine-free. Held(2) reported a marked increase in maternal and cord blood fluorine levels after supplemental fluorine had been given. Feltman and Kosel(3) compared fluorine concentration in cord blood and placental tissue in women drinking fluorine free water with that in women given fluoridated water or fluorine tablets. In both groups placental tissue contained much more fluorine than cord blood. Ziegler(4), using

pooled materials, compared fluorine values in placental tissue, maternal blood and cord blood of a small number of women drinking almost fluorine-free water, with those in women given additional fluorine in milk. In the latter group there was a very significant increase of fluorine concentration in maternal blood and placental tissue; in cord blood the value increased only slightly.

The present study shows a similar relationship in a larger number of subjects, each individually investigated.

Materials and methods. Fluorine determinations were carried out on blood samples taken from 25 healthy, pregnant women shortly before their delivery in the obstetrical department of Hadassah-University Hospital, and from 32 healthy, non-pregnant women. All the subjects lived in Jerusalem, which is supplied by drinking water containing 0.55 ppm fluorine(5). Placentas were obtained at normal deliveries occurring at full term. Net weights of the placentas ranged

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TABLE I. Fluorine Concentration in Placental Tissue, Cord and Maternal Blood.*

Material	No. of cases	F value, ppm		Material compared	Significance of difference
		Mean	Range		
1. Placental tissue	25	.15	.27-.07	1:3	$p < .001$
2. Cord blood	25	.11	.25-.03	1:2	$p < .05$
3. Maternal blood	25	.09	.21-.02	2:3	$p = .075$ (none)
4. Non-pregnant women's blood	37	.18	.36-.04	3:4	$p < .001$

* Maternal blood shortly before delivery.

from 330-795 g. In each case the whole placenta, as well as the blood from the cord after its separation, were analyzed for fluorine content. Examination of the whole placenta was preferred to avoid errors due to a possibly unequal distribution of fluorine in placental tissue(3).

After 20-25 cc of blood and placenta were each made alkaline with fluorine-free Ca (OH)₂ solution (which served also as fluorine fixative), the samples were evaporated to dryness and ashed at about 400°C. The ashed samples were transferred to the distilling apparatus previously described(6) and the chlorides were precipitated with Ag₂SO₄. Fluorine was then steam distilled in presence of H₂SO₄ at 135°-138°C. In the concentrated distillate fluorine was determined colorimetrically by the color fading reaction of a violet iron salicylate complex, as described by Voegtlin and Hodge(7) and Cremer and Voelker(8). The experimental error of this method for blood and placenta samples is ± 0.03 ppm fluorine.

Results. The results are presented in Table I. Mean fluorine values of the placental tissues were significantly higher than in the cord or maternal blood (0.15 ppm, 0.11 ppm, and 0.09 ppm respectively). Mean fluorine content in cord blood was not significantly higher than in maternal blood. Mean fluorine value in the blood of non-pregnant women was significantly higher than that found in the maternal blood before delivery (0.18 ppm and 0.09 ppm respectively). Placenta-cord blood and placenta-maternal blood correlation coefficients were determined in each case. The correlation coefficient between fluorine concentrations in placental tissue and cord blood was significant ($r: +0.5182$). No significant correlation was evident between fluorine concentration in pla-

cental tissue and that in maternal blood ($r: +0.1795$).

Discussion. The significantly lower fluorine level in maternal blood shortly before delivery, compared with that found in the blood of non-pregnant women, corresponds with the decrease of fluorine concentration occurring in urine and saliva of pregnant women(5,9). The significantly higher level of fluorine in the placenta, as compared with that in the maternal blood, indicates that fluorine accumulates in placental tissue. This agrees with the results of Gardner *et al.*(1), and of Ziegler(4). Fluorine accumulation in the placenta does not appear to be related to the actual fluorine concentration found in the maternal blood shortly before delivery. Maternal blood fluorine concentration depends mainly on fluorine content of food and water, and may be subject to daily variations(10, 11). However, due to the amount of blood (about 20-25 cc) needed to determine such variations, repeated estimations were not performed.

Although there was no significant correlation between fluorine in placental tissue and fluorine in maternal blood, in all the cases studied placental concentration of fluorine was always higher than that of maternal blood(4).

The finding that mean fluorine concentration in the placenta is significantly higher than that in cord blood, and that a positive correlation exists between them, indicates that the placenta allows transfer of fluorine to the fetus. The fluorine is utilized mainly for growth development of the bones and teeth of the fetus(12,13,14).

It may be concluded from the above results that fluorine accumulates in the placenta which, at least in regions supplied by drinking water of about 0.55 ppm fluorine,

presents little obstruction to passage of fluorine from maternal to fetal circulation. The fact that concentrations of fluorine in maternal and cord blood are almost the same was considered by Held(2) to imply that the placenta passively permits fluorine transfer to the fetus. Our finding that fluorine concentration in the placenta is higher than in either maternal or cord blood suggests, however, that the placenta plays a more active role.

Summary. Samples of the blood from pregnant women shortly before delivery, placentas at full term and cord blood, were analyzed for fluorine. All subjects drank from the same water source which contained about 0.55 ppm fluorine. The results suggest that the placenta plays an active role in accumulation of fluorine and its transfer to the fetus.

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Carbon Dioxide Requirement and Nucleic Acid Metabolism of HeLa and Conjunctival Cells.* (26267)

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This is the third of a series of publications from this Department on the physiological role of carbon dioxide in multiplying human cells *in vitro*. The following are some of the justifications for carrying out such studies: a) while many compounds are known to have arisen through CO₂ fixation in the heterotrophic organisms, the physiological significance of many such reactions remains obscure; b) the importance of CO₂ in the process of differentiation has been reported(1); c) there are speculations that CO₂ may act as a physiological regulator of cell multiplication(2), and d) very few reports are based on study with intact human cells.

Our first report described CO₂ as an essential for propagation of human cells *in vitro* (3). A subsequent paper demonstrated the role of CO₂ in RNA and protein synthesis as shown by virus formation in human cell culture(4). The present report describes the fixation of C¹⁴O₂ by human cells and recovery of most of the fixed C¹⁴ in the nucleic acids and their derivatives. Under the described experimental conditions, the syntheses of nucleic and oxaloacetic acids are the only demonstrable essential CO₂ fixation reactions of these human cells.

Materials and method. Nutrient media. The tris(hydroxymethyl)aminomethane buffered medium was used exclusively, unless

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otherwise specified, in all experiments. This medium consisted of 10% dialyzed equine serum in Eagle's basal medium(5) with certain modifications(4). No bicarbonate was added and any absorbed CO₂ was minimized by exposing the medium to an alkaline trap for at least 24 hours before each experiment.

Human cell cultures. The HeLa(6) and the conjunctival cells(7) derived respectively from malignant and normal human tissues were used. Stock cultures were propagated and checked at regular intervals for possible contamination by the pleuropneumonia-like organism as described previously(8).

Cell culture method. In all experiments, the cells were grown directly on glass surface of culture tubes (18 × 150 mm) containing 1 ml of nutrient medium and tightly closed with rubber stoppers. The culture was incubated at 36°C on a roller drum of about 12 revolutions per hour. The gentle agitation of nutrient medium in a closed system presumably facilitated the equilibration of metabolites.

Incorporation of C¹⁴ substrates. Twenty-four or 48 hours after addition of C¹⁴ labelled substrates, 2 of a series of replicate cultures were selected at random for cell enumeration. The remaining cultures were rinsed twice with about 20 ml of 0.85% NaCl. The cells were then dissolved in 0.85% NaCl containing 0.02% sodium lauryl sulphate. Cell solutions from 5 to 10 cultures were pooled and then fractionated by the method of Schmidt-

Thannhauser-Schneider(10). Fraction 1 (acid solubles), 2 (lipids), 3 (RNA), 4 (DNA) and 5 (proteins) were obtained. C¹⁴ activities of these fractions were determined. It should be emphasized that any bicarbonate-C¹⁴ present in fraction 1 is presumably expelled as CO₂ during the processing of this fraction for counting. In certain experiments the C¹⁴ activities of the purines and pyrimidines were determined following hydrolysis of whole cells with 7 N perchloric acid(11) and chromatographic separation using isopropanol-HCl as solvent(12).

CO₂ depletion technic. This was accomplished through use of an alkaline trap installed in the gaseous phase of the culture system(3).

Results. C¹⁴O₂ fixation into cell fractions. The results of 5 representative experiments are presented in Table I; more than 90% of C¹⁴ activity resided in the acid soluble and nucleic acid fractions. Such results were consistently observed in 10 experiments, although there were considerable quantitative variations from experiment to experiment. No consistent difference between conjunctival and HeLa cells nourished in serum of equine or human origin have been observed.

C¹⁴ activities of purines and pyrimidines. Following hydrolysis and chromatographic separation of the purines and pyrimidines of cells grown in NaHC¹⁴O₃ containing-medium for 48 hours, C¹⁴ activity was regularly detected in spots formed by guanine, adenine,

TABLE I. Fixation of C¹⁴O₂ into Various Fractions of Conjunctival and HeLa Cells.

Exp.*	Duration of exp. (hr)	Multiplication index†	C ¹⁴ activities‡ in the following fractions				
			1	2	3	4	5
1	24	152/ 96, 193/ 96	151, 165§	64, 16		1630, 1160	126, 120
2	24	278/180, 292/180	280, 231	0, 0		410, 340	114, 165
3	24	222/165, 232/165	370, 330	0, 0		887, 429	281, 156
4	24	112/81	1330	73	322		191 123
	48	187/81	623	26	560		276 78
5	24	115/76	1329	14	335		132 102
	48	162/76	873	15	500		253 105

* The conjunctival cells were used in Exp. 1, 2, 4; HeLa in Exp. 3, 5. Five μ C (5 μ moles) of NaHC¹⁴O₃/10 ml media was used in all experiments. In Exp. 4 and 5, nutrient media were modified by use of double concentrations of amino acids and inclusion of non-essential amino acids at 0.2 mM.

† Denominator and numerator represented No. of cells, in thousands, per culture at 0 and 24 hr (or 0 and 48 hr) respectively.

‡ Expressed as counts/min./million cells; separation into RNA and DNA not attempted in Exp. 1, 2, and 3.

§ Results of replicate sets of cultures.

TABLE II. Multiplication of CO₂-Depleted Cells in Presence of Intermediate Metabolites.

Metabolites added*	Exp. 1		Exp. 2		Exp. 3	
	Cell number,† in thousands, per culture on day					
	7	14	7	14	7	14
None	65, 77‡	19, 18	31, 29	0, 0	17, 26	0, 0
Bicarbonate	649, 643	555, 550	225, 247	217, 184	394, 278	452, 732
Ribosides	314, 297	101, 104	217, 235	250, 232	73, 78	14, 18
Oxaloacetate	80, 95	21, 16	72, 78	8, 7	63, 40	5, 7
Ribosides, oxaloacetate	851, 745	702, 850	488, 405	525	194, 365	170, 398

* Bicarbonate—5 mM, no alkaline trap; ribosides—adenosine guanosine, uridine and cytidine at 10 μ g/ml, and oxaloacetate at 5 mM.

† Zero day counts were 61, 42, and 30 in Exp. 1, 2 and 3 respectively.

‡ Results of replicate culture.

cytosine, uracil and thymine. Total activity of the purines was somewhat higher than the pyrimidines. For example, C¹⁴ activity, expressed as cpm per million cells as 1665, 1143 and 669 for cell hydrolysate, the purines and the pyrimidines respectively in one experiment. In a second experiment, they were 1450, 799 and 584.

CO₂ substitution study. Since the preceding data suggested that most of the CO₂ was utilized for synthesis of the purines and pyrimidines, experiments were designed to determine if these bases were effective substitutes for CO₂ in supporting cell multiplication. A combination of adenine, guanine, cytosine and uracil (with or without thymine) at concentration of 0.1-10 μ g per ml media was found ineffective as CO₂ substitute. Adenosine, guanosine, cytidine and uridine at similar concentrations were partially effective; evidence of partial degeneration was invariably present in all experiments. If oxaloacetate was added, in addition, cell multiplication occurred at a rate at least equal to that in the presence of CO₂. Oxaloacetate, without the ribosides, was ineffective. Malate, with or without the ribosides, was also ineffective. Results of 3 representative experiments are presented in Table II. Occasionally, the accumulation of acid metabolites occurred at such rapid rate that great difficulty was encountered in maintaining the pH of the media at 7.2-7.8. Such difficulty may be readily remedied by replacing glucose in the nutrient medium with galactose. Experiment 1 was performed with the glucose medium, and 2 and 3, the galactose medium. The following metabolites were ineffective substi-

tutes for CO₂ in supporting the multiplication of the conjunctival cells: 1) the deoxyribosides at 10 μ g per ml; 2) glycine, serine, alanine, aspartic acid, asparagine and glutamic acid at 0.2 mM; 3) oleic acid at 10 μ g per ml; 4) creatine at 0.1 mM; 5) uridine at 10 μ g per ml, or 6) adenosine at 10 μ g per ml.

Discussion. Our present study of the significance of CO₂ fixation may be compared with similar studies of the bacteria, particularly those of Wilson and his associates(13, 14, 15), Griffin and Racker(16) and Tuttle and Scherp(17). When *Brucella abortus* were grown in the presence of C¹⁴O₂, most of the activity was found associated with glycine and other amino acids. Only 6% of C¹⁴O₂ fixed was recovered in the nucleic acids of the *Brucella* and almost all this activity was found in the pyrimidines. No substitutes capable of replacing CO₂ have yet been found for *Brucella*. With *Neisseria meningitidis*, no substitute for CO₂ has been found, although a combination of guanine, uracil, cytosine, casein hydrolysate and the B vitamins was partially effective. In the case of *Neisseria gonorrhoeae*, again only a partial substitute for CO₂ may be found in a combination of hypoxanthine, uracil and oxaloacetate. Our results with human cells differed significantly in that most of the C¹⁴O₂ fixed by the cells was recovered in the nucleic acids and their purine and pyrimidine derivatives, and that a combination of the ribosides and oxaloacetate was found completely effective as a substitute for CO₂ in supporting cell growth. These findings are in general agreement with Geyer and Niemark who showed that cell extract was an effective substitute

for CO₂(9); their results suggested a relationship between CO₂ and biosynthesis in these cells. Of interest was a recent abstract reporting on recovery of C¹⁴ activity in the purines, pyrimidines and certain amino acids of mouse cells grown in presence of C¹⁴O₂ (22).

The completeness of CO₂ depletion in our experiments may be questioned. Since our previous report(3) showed that CO₂-depleted cells continued to oxidize glucose-1-C¹⁴ to C¹⁴O₂, a very low level of CO₂ must still be present. That different levels of CO₂ depletion may give rise to different results is shown by the several reports using various methods of depletion. For instance, Eagle, replacing the bicarbonate component of his nutrient medium by tris found that a massive culture multiplied adequately in a closed system(18). Swim and Parker found conditions described by Eagle inadequate if an open tissue culture system was used (19); presumably, endogenous CO₂ cannot accumulate. Gwatkin and Siminovitch(20), working with small cell populations in an open system, found oxaloacetate an adequate substitute for CO₂. By use of an alkaline trap in a closed system, a method first used by Harris for chick tissue(21), the ribosides and oxaloacetate were both essential. If a practical method for further depletion of CO₂ is available, it should not be too surprising if some other metabolites may also be found essential.

The syntheses of the purines, pyrimidines and oxaloacetate from CO₂ are some of the many well established CO₂ fixation reactions in lower forms of life. Our data are significant, not in demonstration of the existence of similar reactions in human cells, but more in the following findings: 1) these CO₂ fixation reactions are essential for survival and multiplication of human cells; 2) among a large number of CO₂ fixation reactions, the syntheses of the purines, pyrimidines and oxaloacetate are the only ones demonstrated essential, and 3) synthesis of the nucleic acid precursors is quantitatively the most significant CO₂ fixation reaction.

Summary. Over 90% of C¹⁴ fixed by HeLa or conjunctival cells growing in a tris-

buffered nutrient medium containing 5 microcuries of NaHC¹⁴O₃ per 10 ml was recovered in the acid soluble and nucleic acid fractions. Most of this activity was found associated with the purines and the pyrimidines of such cells. The ribonucleosides were partially effective as a substitute for CO₂ in supporting cell multiplication. A combination of ribosides and oxaloacetate provided conditions as good as or better than CO₂ for multiplication of CO₂-depleted conjunctival cells. It may be concluded that, under the described experimental conditions, the chief functions of CO₂ in human cells are to provide specific precursors for syntheses of the purines, the pyrimidines and oxaloacetate.

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Effect of ACTH and Insulin on AIB Accumulation in Diaphragm Muscle and Adrenal *in vivo*.^{*†} (26268)

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It has been shown previously that adrenocorticotropin (ACTH) produces changes in sugar permeability in the adrenal which are similar to those produced by insulin in muscle(1,2). In both these tissues of the hypophysectomized rat, the "non-utilizable" pentose, D-xylose, is excluded from a major fraction of the cell water; following ACTH administration the intracellular distribution of D-xylose is markedly increased in adrenal but not in muscle, whereas insulin acts upon sugar transfer in muscle but not adrenal. In addition, insulin also increases amino acid transport in isolated rat diaphragm, as evaluated by use of the "non-utilizable" amino acid α -aminoisobutyrate (AIB)(3). To study further the effects of these hormones on amino acid transport, the present work examines the distribution of AIB in the adrenal and diaphragm of functionally-nephrectomized rats before and after hypophysectomy and of hypophysectomized-nephrectomized rats receiving exogenous ACTH, insulin, corticosterone, or growth hormone.

Methods. To establish appropriate experimental conditions, the distribution of AIB between tissues and plasma was determined at 2 time intervals after administration of AIB, and at a variety of plasma concentrations of AIB. Intact or hypophysectomized (45-50 hr) rats of the Sprague-Dawley strain weighing 120-160 g were used. The renal pedicles were ligated under avertin anesthesia immediately before subcutaneous injection of α -AIB-1-C¹⁴ (Volk). This route of AIB administration and functional nephrectomy was employed to achieve a relatively constant plasma concentration during the experiment. Qualitatively similar, but more variable re-

sults were obtained when the kidneys were not ligated. Dosage and specific activity of AIB was varied to achieve a range of plasma concentrations extending from 0.3 to 850 μ g/ml.

AIB determinations were carried out on adrenal, diaphragm, and plasma either 1.5 or 5 hr after AIB administration. After these intervals, the rats were reanesthetized (nembutal), blood was drawn from the aorta into a heparinized syringe. While plasma was being obtained without delay by one of us, others excised the tissues immediately, which were extracted for AIB with hot water in a boiling water bath for 15 min, as employed previously(1). Results obtained by this extraction method were similar to those obtained using the acetic acid-homogenizing method of Noall *et al.*(4). Moreover, it was established that after extracting tissues with hot water, no significant amount of radioactivity could be extracted following homogenization in water or acetic acid; the boiling water procedure extracts much less solid material from tissues than the acetic acid homogenization method, and consequently self-absorption is less in the former method. Tissue extracts and diluted plasma (1:50) were plated and assayed for radioactive content in a windowless flow gas chamber.

To determine whether the radioactivity extracted and measured in this manner is unmodified AIB, pooled extracts from adrenal, diaphragm, and liver were treated as follows. The radioactivity was separated from the bulk of the non-radioactive tissue extractives by applying the aqueous extracts to Whatman #1 paper, and eluting the radioactivity using 95% EtOH as mobile phase. The ethanolic overflow collected for 2-3 days contained all of the radioactivity, and was then chromatographed in the n-butanol: water:acetic acid system (5:5:1) for 8 hours. In all cases, only one radioactive peak of R_f value corresponding to pure AIB was ob-

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served. Quantitative elution of the paper revealed that of the total count recovered, 86-90% was present in the AIB zone from adrenal tissue, and 98-100% in those zones from liver, and diaphragm. The data indicate that radioactivity extracted from these tissues primarily represents unchanged AIB, but does not exclude the possibility that a small fraction of the AIB accumulated by adrenal has been modified.

Considering, therefore, that metabolism of AIB does not occur to a significant extent, distribution of AIB between tissues and plasma in these experiments is expressed directly as a ratio, counts per min per g wet tissue/counts per min per ml plasma. AIB distribution in intracellular water (AIB ml cell water/AIB ml plasma) was calculated by measuring inulin space as described previously(1) and by determining total water per g tissue. Total water for the tissues was determined by drying to constant weight at 105°. The intracellular fluid volume was assumed to be the difference between total water and inulin space, assuming that inulin distributes in the extracellular fluid exclusively, and is equilibrated with the plasma.

In those cases where the effect of exogenous hormones was tested, the hormones were administered to hypophysectomized rats just after AIB was administered subcutaneously; in all cases AIB distribution values were obtained 90-100 min after AIB was given in an amount necessary to achieve AIB plasma concentrations ranging from 0.3 to 10 $\mu\text{g/ml}$. The hormones studied, with the dosage per 100 g body weight, were as follows: 375mU corticotropin A (Armour) given as a single intravenous injection; 30 μg bovine growth hormone (Li), given intravenously; corticosterone, 0.7 mg in a divided dose in 10% alcohol, one-half given intravenously and the remainder subcutaneously; and 1 U insulin (Iletin, Lilly) injected intraperitoneally. In the experiments with insulin, to prevent hypoglycemia, 100 mg glucose per 100 g body weight was injected along with the insulin; controls for this group received 25 mg glucose per 100 g body weight.

Results. Fig. 1 graphically presents the results obtained in adrenal and diaphragm of

functionally nephrectomized rats at the 2 time intervals employed, 1.5 and 5 hr. Distribution ratio of AIB in these tissues of both hypophysectomized and pituitary-intact rats is given at plasma concentrations of AIB ranging from about 0.3 to 850 $\mu\text{g/ml}$. It is clear that hypophysectomy alters distribution of AIB in both tissues in 2 respects. First, level of AIB uptake is reduced in the absence of the pituitary, the extent of the difference being dependent on plasma AIB concentration. Secondly, the tissues of hypophysectomized rats differ from intact in response to increases in plasma AIB concentration. In the hypophysectomized rat, uptake of AIB by these tissues increases in proportion to plasma concentration so that in this case the AIB distribution ratio is relatively constant over the entire range of plasma concentrations studied. In the intact rat, this constancy of distribution appears to be manifest only at the lower levels of plasma AIB concentration, below 10 $\mu\text{g/ml}$. Above this concentration, in contrast to the findings in the hypoxed rat, AIB distribution declines as plasma levels are progressively increased, so that at plasma concentrations of about 800-850 $\mu\text{g/ml}$, distribution of AIB in these tissues is reduced to the level observed in the hypophysectomized rats. At the lower plasma concentrations of AIB, to about 10 $\mu\text{g/ml}$, the decline in distribution after hypophysectomy is nearly identical in both tissues, being 41-44% of the values in the intact.

Table I presents the average of the tissue distribution values obtained in both groups at plasma concentrations of AIB less than 10 $\mu\text{g/ml}$, where AIB distribution values in both groups were independent of plasma AIB level. Table I also presents the calculated ratio of AIB concentration in intracellular to extracellular water. It is seen that in the adrenal the ratio of $\text{AIB}_{\text{in}}/\text{AIB}_{\text{out}}$ is 2.9 at both time intervals in the hypophysectomized rat, while this ratio is 7.0 and 7.3 at 1.5 and 5 hr, respectively, in the pituitary intact rat, indicating that in the adrenal the accumulation of AIB attains a steady state by 1.5 hr. In diaphragm, however, maximal distribution was not achieved in 1.5 hr; intracellular distribution ratio increases from 0.8 at 1.5 hr

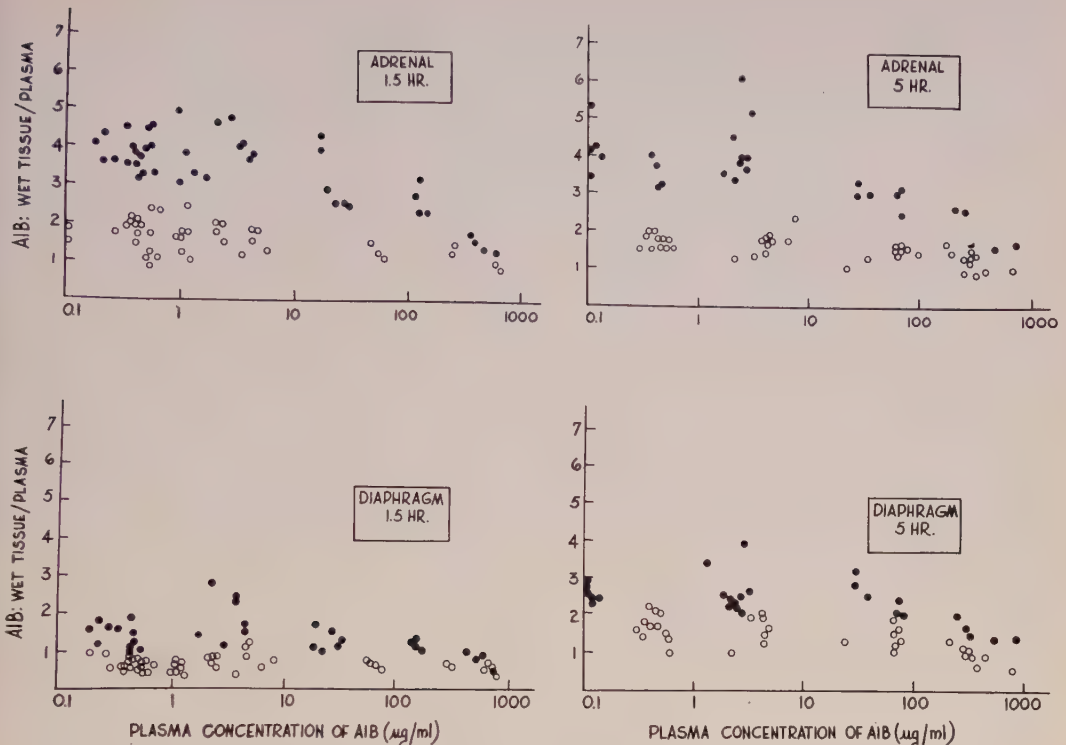


FIG. 1. Distribution ratio of AIB (counts per min. per g wet tissue/counts per min. per ml plasma) for adrenal and diaphragm muscle of hypophysectomized (○) and pituitary intact rats (●) at 1.5 or 5 hr after inj. of AIB, plotted against plasma concentration of AIB (abscissa, logarithmic scale).

to 2.5 at 5 hr in hypophysectomized, and from 2.3 at 1.5 hr to 4.1 at 5 hr in pituitary intact rats. The relationship of the pituitary intact to the hypophysectomized group, and qualitative pattern of AIB distribution, however, are similar at both time intervals.

The effects on AIB transport by various hormones were studied at AIB plasma levels of 10 μg/ml or less because: (a) AIB distribution ratios in adrenal and diaphragm of both hypophysectomized and pituitary-intact

rats remain relatively constant, when AIB plasma level is within this range; thus any changes in plasma AIB concentrations associated with a hormonal effect would have minimal influence on AIB distribution ratio and (b) the differences between hypophysectomized and pituitary-intact rats are most clearly revealed in this range, and diminish as AIB plasma level is progressively increased. The effects of various hormones on AIB distribution in the adrenal and dia-

TABLE I. Distribution of AIB between Tissues and Plasma of Intact and Hypophysectomized Rats, 1.5 and 5 Hr after AIB Administration.

Time interval (hr)	Type animal	% distribution of AIB* [(wet tissue/plasma) × 100]		Distribution ratio of AIB (intracellular water/plasma)	
		Adrenal	Diaphragm	Adrenal	Diaphragm
1.5	Intact	400 ± 10 (28)	157 ± 12 (18)	7.0	2.3
	Hypox.	177 ± 8 (34)	65 ± 4 (36)	2.9	.8
5	Intact	417 ± 19 (18)	266 ± 11 (16)	7.3	4.1
	Hypox.	175 ± 6 (21)	166 ± 9 (18)	2.9	2.5

* Mean, stand. error, and No. of observations, obtained at plasma conc. of AIB less than 10 μg/ml.

TABLE II. Influence of Various Hormones on Distribution of AIB in Adrenal and Diaphragm of Functionally-Nephrectomized, Hypophysectomized Rats.

Treatment (dosage/100 g body wt)	% distribution of AIB (total wet tissue)*	
	Adrenal	Diaphragm
Saline (0.9%)	177 $\pm$ 8 (33)	65 $\pm$ 4 (36)
Corticotropin A, Armour (375 mU)	298 $\pm$ 23 (10)	90 $\pm$ 8 (8)
Growth hormone, Li (30 μ g)	176 $\pm$ 8 (5)	54 $\pm$ 5 (5)
Alcohol (10%)	178 $\pm$ 8 (5)	57 $\pm$ 7 (5)
Corticosterone (0.7 mg in 10% alcohol)	114 $\pm$ 14 (5)	40 $\pm$ 6 (5)
Glucose (25 mg)	192 $\pm$ 14 (5)	72 $\pm$ 7 (5)
Insulin (1 U), glucose (100 mg)	291 $\pm$ 11 (16)	420 $\pm$ 110 (16)
Insulin (1 U) + Corti A (375 mU)	429 $\pm$ 18 (5)	510 $\pm$ 69 (5)

* Values are mean, stand. error, and No. of observations, obtained 1.5 hr after administration of AIB and hormones, at plasma concentration of AIB less than 10 μ g/ml.

phragm of functionally-nephrectomized, hypophysectomized rats obtained under these conditions are shown in Table II. Intravenous administration of corticotropin A significantly increased distribution in both tissues, giving values 68% above hypophysectomized in the adrenal ($p = <.001$) and 38% higher in diaphragm ($p = <.01$). Growth hormone, in the dosage used (30 μ g/100 g) had no effect on AIB distribution in these tissues of the hypophysectomized rat. Corticosterone reduced distribution in adrenal 36% below that of the alcohol control ($p = <.01$), and tended to reduce values in diaphragm. With insulin, adrenal distribution increased 52%, and diaphragm increased 480% ($p = <.001$, in both cases). When corticotropin A was given together with insulin, a further significant increase in adrenal occurred ($p = <.001$) giving an average value equivalent to that in the intact. This combined treatment also tended to elevate distribution in diaphragm above that with insulin alone, but this change is not statistically significant.

Discussion. The present findings describing the effects of insulin and ACTH on transport of AIB in adrenal and diaphragm muscle of hypophysectomized rats contrast sharply in 2 respects with previous studies on sugar

permeability as evaluated with D-xylose(1). First, D-xylose was excluded from a major fraction of the cell water of both adrenal and diaphragm, whereas AIB is accumulated against an apparent concentration gradient in both tissues. Secondly, the hormones specifically increased D-xylose transfer in their target organs; ACTH influenced adrenal but not muscle, while insulin affected muscle but not adrenal. With AIB, on the other hand, either ACTH or insulin produce increased accumulation of AIB in both adrenal and diaphragm muscle. The responses in adrenal to ACTH and insulin are equivalent, but the increase in muscle AIB accumulation caused by insulin is greater than that produced by ACTH. The mechanisms of sugar transfer and amino acid transport are obscure, and accordingly the basis of the observed differences in hormone specificity cannot be evaluated. Part of the difficulty may relate to the fact that the adrenal represents 2 tissues and the effects observed may involve either cortex or medulla, or both. While it is reasonable to expect that ACTH acts on the adrenal cortex, the locus of insulin action in the present adrenal studies remains unresolved. However, it may be significant that insulin is reported to increase adrenal gland weight of hypophysectomized rats(5) and pigeons(6); in the latter case, the changes caused by insulin were more pronounced in the cortex and were cytologically identical to those produced by ACTH.

The study with growth hormone indicates that it is unlikely the observed effects of ACTH were due to trace amounts of growth hormone in the preparation employed, since growth hormone alone, injected under conditions identical to ACTH, had no effect on AIB levels in adrenal or diaphragm muscle of hypophysectomized rats.[†] Similarly, the results with corticosterone suggest that the stimulatory effect of ACTH was not due to

[†] The conditions employed in this study are not necessarily optimal for studying the effect of growth hormone on AIB transport, since effects might have been obtained had different conditions been employed. Thus, it has been reported that growth hormone added *in vitro* increases AIB uptake by diaphragm muscle excised from rats hypophysectomized 14 days or longer(7).

a secondary effect of corticoid released under ACTH stimulation, since corticosterone significantly inhibited AIB uptake in adrenal and tended to lower AIB in diaphragm. Nor does it seem probable, in view of the results with corticosterone, that the effects of ACTH are due to stimulation of the release of endogenous insulin by corticoids released following ACTH administration. This is supported by the fact that ACTH given under similar conditions had no effect on D-xylose transfer into diaphragm muscle(1).

It should be noted that a single large intravenous dose of corticotropin A does not restore adrenal AIB distribution to that found in pituitary-intact rats. This might be due to the particular mode of administration of ACTH employed in this study, to a decreased responsivity of the adrenal to ACTH following hypophysectomy, or to lack of an additional factor, which could be either pituitary or extra-pituitary in origin. The latter possibility should be considered since it was found that when insulin was given together with ACTH to hypophysectomized rats, AIB distribution was raised to the pituitary-intact level.

The significance of studies with AIB is related to the assumption that the behavior of AIB serves as an index of the transport of natural amino acids, and further that a change in the availability of amino acid to the interior of cells may influence protein synthesis and/or amino acid metabolism. On this basis, the present studies suggest that ACTH increases the uptake of amino acids by adrenal cells, and that this effect may be

important in the increased protein synthesis and growth caused by ACTH in adrenal cortex. Insulin also appears to be involved in amino acid transport in adrenal as well as muscle, thus providing additional evidence that this hormone may have a role in adrenal maintenance and growth.

Summary. Hypophysectomy causes a decline in accumulation of the amino acid analogue, α -aminoisobutyrate (AIB), in adrenal and diaphragm muscle of functionally nephrectomized rats. Either Corticotropin A or insulin increase AIB accumulation to an equivalent extent in adrenal of hypophysectomized rats; both hormones also increase AIB uptake in diaphragm muscle, but insulin is more effective in this tissue. The combined influence of these hormones on both tissues is greater than either alone. The significance of these observations for protein synthesis and growth in the adrenal are discussed.

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Observations on Effect of 3-Methylcholanthrene in Scorbatic Guinea Pigs. (26269)

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Previous studies have demonstrated a marked increase in synthesis of L-ascorbic acid following administration of 3-methylcholanthrene (3-MC) to rats(1,2). Since

guinea pigs lack the ability to synthesize L-

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ascorbic acid, it was thought of interest to test the response of scorbutic guinea pigs to this compound. Observations presented here indicate that administration of 3-MC reverses some of the signs of scurvy in guinea pigs without inducing any detectable L-ascorbic acid formation.

Methods and materials. The modified curative bioassay for Vit. C activity employed here was similar to that used previously (3). One hundred and thirty male guinea pigs weighing about 200 g were given a Vit. C free diet[†] for 21 days. After 14 days on the diet they were divided into the following groups and received the indicated treatment for the 7 remaining days: Group 1 was given 1.0 mg of L-ascorbic acid orally per day; Group 2, 1.0 mg of 3-MC in 0.5 ml corn oil per day by i.p. injection; Group 3, 3.0 mg of 3-MC in 0.5 ml corn oil per day by i.p. injection; Group 4, 5 mg of 3-MC in 0.5 ml corn oil by i.p. injection; Group 5, 10 mg of 3-MC in 0.5 ml corn oil per day by i.p. injection, and Group 6, 0.5 ml of corn oil per day by i.p. injection (negative controls). The animals were weighed daily and were sacrificed on the 21st day and the knee joints examined for gross signs of hemorrhage. The lower jaws from 7 or more animals in each group were immediately fixed in Lillie's (5) aqueous neutral calcium acetate 10% formalin solution for 24 hours, decalcified in 5% formic acid, dehydrated, embedded in paraffin and sectioned mesiodistally at 6 μ . Sections were stained with hematoxylin and eosin, the Rinehart stain (6), azure A (0.5% in aqueous phosphate buffer pH 4, 10 minutes, 25°C), and the alloxan-Schiff stain (7).

The tissue concentration of L-ascorbic acid was determined by the method of Roe and Kuether (8). The possibility that 3-MC induced synthesis of L-ascorbic acid was investigated by an isotopic procedure used previously (9,10). Ten milligrams of D-galactose-1-C¹⁴ with a specific activity of 4.72 μ c/mg was administered on the 20th day of experiment to each of 2 guinea pigs receiving 10 mg

TABLE I. Incidence and Extent of Hind Knee Hemorrhages in Guinea Pigs Fed a Vit. C Free Diet with and without Supplements of L-Ascorbic Acid or 3-Methylcholanthrene.

Group*	Supplement	Extent of hemorrhage			
		None	Slight	Moderate	Severe
1	1 mg L-ascorbic acid	21	1	1	
2	1 mg 3-MC	12	3	3	1
3	3 " "	4	2	1	
5	10 " "	18	2		
6	None	1	5	12	14

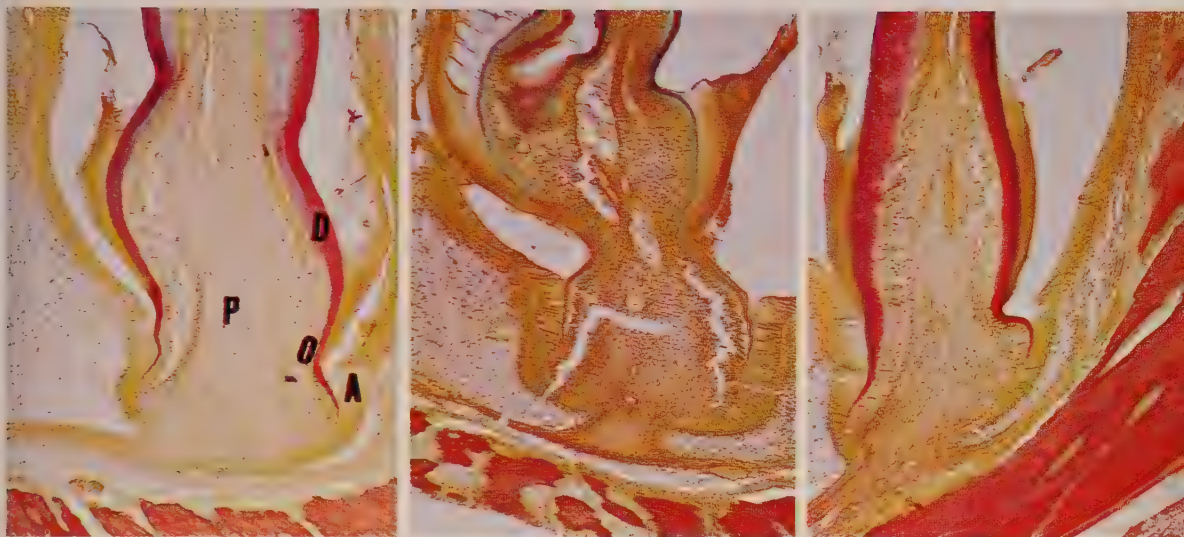
* Description of the various groups is contained in section on methods.

of 3-MC (from Group 5). Twenty-four hours later the animals were sacrificed and liver, spleen, testes, kidneys and adrenals from each animal were pooled and homogenized in 4 times their weight of 5% trichloroacetic acid. L-Ascorbic acid was isolated from the extract after addition of 200 mg of carrier by ion exchange chromatography, converted to its 2,4-dinitrophenylosazone derivative and recrystallized successively from an acetone-alcohol mixture. Radioactivity in the derivative was assayed in a gas flow counter. The C¹⁴ in carbon 6 of L-ascorbic acid was determined by periodic acid cleavage of the primary alcohol group to give formaldehyde which was recovered and counted as its dimedon derivative.

Results. Gross observations. The animals initially gained weight until about the 11th day on the diet when their weight began to decline. On the 15th day the animals were treated as described in the methods section. Animals in Group 1 gained an average of 10 g from the 15th to the 22nd day. During this period the animals in the negative control group as well as those receiving 3-MC lost about 20 g. Survival of animals in the various groups was essentially the same.

Incidence of gross hemorrhages of the knee joints in the various groups is listed in Table I. Administration of 3-MC to scorbutic guinea pigs markedly reduced incidence and extent of hemorrhages. This was particularly evident in those animals receiving the 10 mg dosage. *Histochemical observations.* In Plate 1A and 1D are shown photomicrographs of sections of molar teeth apices procured from the animals treated with L-ascorbic acid

[†] The vit. C free diet, prepared as described by Woodruff *et al.* (4), was purchased from Nutritional Biochemical Co., Cleveland, O.



(Left to right) *a.* Apex of a lower molar from a guinea pig treated with L-ascorbic acid (Group 1). Rinehart stain. Dentin at apex and that nearest pulp was produced last. Red stain indicates collagen, dentin or bone. Blue stain indicates mucopolysaccharide. D, dentin; P, pulp; O, odontoblasts; A, ameloblasts. $\times 50$. *b.* Apex of a lower molar from a guinea pig in negative control group (Group 6). Rinehart stain. Note that very little dentin was produced during scorbutic state; and the strong reactivity of stain for mucopolysaccharide in abnormal osteodentin and connective tissue generally. $\times 50$. *c.* Apex of a lower molar from a guinea pig treated with 10 mg of 3-MC daily for one week (Group 5). Rinehart stain. Note restoration of normal odontogenesis and lack of hemorrhages in the pulp. $\times 50$.



(Left to right) *d.* Apex of a lower molar from a guinea pig treated with L-ascorbic acid (Group 1). Ninhydrin-Schiff stain. Pink and red stain indicates reactive protein. $\times 50$. *e.* Apex of a lower molar from a guinea pig in negative control group (Group 6). Ninhydrin-Schiff stain. Note lack of reactive protein in dentin produced during scorbutic state. $\times 50$. *f.* Apex of a lower molar from a guinea pig treated with 10 mg of 3-MC daily for one week. Ninhydrin-Schiff stain. Note restoration of normal protein reactivity. $\times 50$.



(Group 1). Note the preservation of the odontoblasts and ameloblasts, and the regularly oriented tubular dentin which stains red with the Rinehart stain (1A), and pink with the ninhydrin-Schiff method (1D). Animals in this group also demonstrated abundant acid mucopolysaccharide exhibited by its metachromasia with azure A in their gingivae.

The jaws of guinea pigs in the negative control group (Group 6) showed the hemorrhages, bone resorption and interruption of dentinogenesis which are characteristic of the disease (1B). Analysis of sections stained with the ninhydrin-Schiff method (1E) corroborated our previous observations(3) that dentin and bone, produced during the scorbutic state, are unreactive with the alloxan-Schiff method for proteins. The gingivae usually failed to induce metachromasia with azure A, but occasionally a very slight metachromasia was exhibited.

The periodontia of animals to which 10 mg of 3-MC had been administered (Group 5) usually did not manifest histological or histochemical evidence of scurvy. Administration of 10 mg 3-MC not only prevented the characteristic destruction of those odontoblasts situated at the onset of dentinogenesis, but preserved their integrity and metabolic function to the degree that apparently normal dentin was produced as evidenced by the normal reactions of the Rinehart (1C) and ninhydrin-Schiff (1F) stains. The widely dilated blood vessels and hemorrhages in the pulp tissues characteristic of scurvy were alleviated in the 10 mg 3-MC treated group to such a degree that normal or near-normal blood vessels without hemorrhage were usually observed (1C). Metachromasia frequently appeared to be fully restored in the gingivae of animals treated with 10 mg 3-MC, and bone resorption also appeared to be arrested. Animals that received lesser amounts of 3-MC (Groups 2, 3 and 4) manifested scurvy to some degree but in general the various pathoses were noticeably reversed.

Ascorbic acid synthesis and retention. Since 3-MC is known to increase the rate of L-ascorbic acid formation when administered to rats, the possibility that it induced synthe-

TABLE II. Conversion of D-Galactose-1-C¹⁴ to L-Ascorbic Acid-6-C¹⁴ in Rats and 3-MC Treated Guinea Pigs.

Animal	% conversion to tissue L-ascorbic acid*
Rat 1	.044
" 2	.066
Guinea pig 1	<.0004
" " 2	<.0003

* L-ascorbic acid was isolated by a carrier dilution technic(9) from a pooled sample of liver, spleen, kidney, adrenal and testes 24 hr after a dose of D-galactose-1-C¹⁴. Guinea pigs used in this experiment had received 10 mg dosage of 3-MC (Group 5). Data obtained in rats have been published(10).

sis of the vitamin in guinea pigs was investigated.

Levels of L-ascorbic acid in brain, liver, testis, eye, intestine, muscle and adrenal were measured in scorbutic animals (negative control group) and in animals receiving 10 mg of 3-MC per day (Group 5). The values were found to be low and to fall within the same range. Levels of L-ascorbic acid in brain and liver from animals receiving 1.0 mg L-ascorbic acid per day (Group 1) were found to be elevated above those in the scorbutic animals (Group 6) from 7.0 to 15.8 mg % in brain and from 1.0 to 3.0 mg % in liver. More definitive evidence against synthesis of L-ascorbic acid in 3-MC treated guinea pigs was obtained by introducing D-galactose-1-C¹⁴ into the carbohydrate pool from which L-ascorbic acid is readily synthesized in rats. Conversion of D-galactose-1-C¹⁴ to L-ascorbic acid-6-C¹⁴ in rats averaged 0.05% whereas less than one-hundredth this value was observed under the same conditions in 3-MC treated guinea pigs (Table II).

Discussion. Administration of 3-MC reduced markedly the incidence of joint hemorrhages associated with scurvy but loss of weight was not prevented. It is not known whether the weight loss was part of the scorbutic syndrome or due to toxicity of the 3-MC.¶ Histological data indicating that 3-MC reverses some of the pathoses of scurvy include restoration of mucopolysaccharides in

¶ The latter may be the case since administration of 1 mg of 3-MC per day for 4 days to normal guinea pigs was found to induce a 20 g weight loss.

the gingivae, preservation of the odontoblasts to a metabolic state whereby normal dentin was produced, and cessation of bone resorption. In most cases the jaws of guinea pigs receiving the 10 mg dosage of 3-MC were indistinguishable from those animals receiving the L-ascorbic acid supplement.

Chemical analysis of L-ascorbic acid in various tissues from Groups 1, 5 and 6 indicated that 3-MC does not alter retention or synthesis of L-ascorbic acid. Experiments utilizing D-galactose-1-C¹⁴ virtually exclude the possibility of 3-MC induced L-ascorbic acid synthesis in scorbutic guinea pigs at least through known pathways(11).

The mechanism by which 3-MC reverses some of the signs of scurvy is not known. Other studies have shown that 3-MC exerts a stimulatory effect on protein synthesis and on the activity of certain enzyme systems(12, 13, 14, 15, 16). It is possible that some of the pathoses in scurvy result from impaired synthesis of certain proteins and/or a lessened activity of certain enzymes which are corrected in part by 3-MC.

Summary. 1. Some of the pathoses of scurvy in guinea pigs were reversed by administration of 3-methylcholanthrene. These included a marked reduction of incidence of hemorrhage, reduction of bone resorption, preservation of dentinogenesis and prevention of death of odontoblasts. 2. The effect 3-methylcholanthrene exerts upon scurvy does not appear to be mediated by induction of L-ascorbic acid synthesis nor by increased retention of the vitamin.

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Localization of Proelastase in the Goose-Fish (*Lophius piscatorius*).^{*} (26270)

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It has been suggested(1,2) that elastase (3) is localized in the pancreatic islets of Langerhans. These observations were made

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before the existence of proelastase(4) had been recognized. As a consequence very little activity could be measured. In view of the possible role of elastase in the etiology of atherosclerosis(5) it was thought of interest

TABLE I. Proelastase Assay of a Mesenteric Acinar Tissue Extract.

Elastin, mg	Extract, ml	Trypsin, ml	Buffer, ml	O.D.
50	.5	1	1.5	.604
50	.5	—	1.5	.127
—	.5	—	1.5	.137
50	—	1	2.0	.055
—	—	—	3.0	Blank

to determine the location of the proenzyme in the pancreas.

The Goose-fish, *Lophius piscatorius*, is a choice animal for such studies because the exocrine and endocrine portions of the gland are anatomically separated(6,7,8). The acinar portion is mainly located as bands of tissue in the mesentery, usually along the branches of the portal vein, while the islet portion is localized in major part as a single mass of tissue (principal islet of Rennie) near the origin of the cystic duct. In the capsule of the principal islet, however, several lobules of acinar tissue can be found. These are easily dissected by removal of the capsule which is attached to the islet only by loose connective tissue.

Approximately 500 Goose-fish were collected and dissected upon arrival at the laboratory. Three different fractions were separated and stored frozen for subsequent activity determinations: mesenteric acinar tissue, capsule of the principal islet and the central endocrine portion of the islet.

Determination of proelastase depends upon its activation by trypsin(4). The hydrolytic activity of the elastase produced is then measured, using a standardized preparation of elastin as substrate(9). The first step of the reaction is so rapid (~1 min) that it is carried out simultaneously with the second step.

Table I gives a typical example of a proelastolytic assay carried out on mesenteric acinar tissue. The capsules were ground in a hand homogenizer with Tris buffer 0.06 M, pH 9.15. The homogenate was lightly centrifuged to remove the strands of connective tissue and the supernatant used as the source of proenzyme. Six flasks were prepared as shown in the table. Trypsin was used at a concentration of 0.5 mg/ml dissolved in the

standard Tris buffer. It has been shown(9) that amount of elastase produced is proportional to concentration of trypsin until all the proelastase is activated. The concentration of trypsin used in these assays is about twice that necessary completely to activate 0.5 ml of a raw extract of acinar pancreas containing approximately 50 mg protein. Since Goose-fish elastase has an optimum pH in the range 9.1-9.5 similar to that of rat and hog elastase(9), all assays were performed at pH 9.15. After 20 minutes incubation at 37° the reaction was stopped by 2 ml of phosphate buffer, 0.7 M, pH 4.5 and 5 ml of water was added to each flask. The unhydrolyzed elastin was centrifuged out and a Lowry protein determination(10) was performed on 0.2 ml of the supernatant. The resulting optical density was read at 750 μ .

The optical densities given in rows 2 and 3 (Table I) are similar. Without trypsin no elastolytic activity exists in the extract. These optical densities are a measure of protein concentration in the extract. The difference between optical densities in row 1 and those in rows 2 and 4 gives the optical density due to the elastin which has been hydrolyzed under the influence of the activated proelastase. This value can be converted to milligrams of elastin hydrolyzed by means of the relation established previously (9): O.D. = $0.0248 \times (\text{mg elastin hydrolyzed})$.

Concentrations of proelastase in the 3 ana-

TABLE II. Proelastase Activity in Tissue Extracts of Different Tissues.

Extracts	mg elastin dissolved in 20 min./100 mg dry tissue
Rat pancreas	19.7
Goose-fish islet	
Acinar tissue	83
Goose-fish mesenteric	
Acinar tissue	196-394
Goose-fish islet tissue (stored frozen)	0
Goose-fish islet tissue (fresh)	0
Cellular islets granules	
(a) whole	0
(b) treated with detergent	0
(c) " " trypsin	0

tomical fractions of the pancreas of the Goose-fish are reported in Table II. All activity is concentrated in the acinar tissue and is greater, on a specific basis, than that found in rat pancreas. The mesenteric fraction seems to be more active than the capsular fraction, probably because of the larger amount of connective tissue present in the capsule. The islet tissue was tested under a variety of conditions: fresh tissue, repeatedly frozen and thawed tissue, granular and supernatant fractions obtained by light centrifuging of the raw extract, as well as granular fractions disrupted by detergent or trypsin. In all cases no proelastolytic activity could be detected.

Since one of the earlier observations(1) describing elastase activity in the endocrine portion of the islet was made by examining elastin fibers under the microscope after 24 hours incubation with the extract, we have performed an assay under similar conditions. We found that 3.5 mg of elastin had been digested, an amount corresponding to 0.2 mg of elastin digested in 20 minutes per 100 mg of tissue. This negligible amount may be due either to a slight contamination by the

capsular proelastase or to bacterial contamination.

We conclude that in the Goose-fish, elastase and its precursor proelastase are produced in the exocrine pancreas, a location typical of a proteolytic enzyme.

Summary. A proelastase resembling that of the pig and the rat has been found in the pancreas of the Goose-fish. It is localized in the acinar portion of the pancreas, both in the mesenteric bands and in the capsule of the principal islet. However it is absent from the endocrine portion of this organ.

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Pasteur Reaction and Glycogen Content of Rat Brain After Noble-Collip Drum Shock.* (26271)

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Shock produced by any method results in systemic hypoxia. Several investigators have based their work on the influence of shock upon intracellular processes on this fact. They excised tissue from normal animals and subjected it to anoxic conditions, e.g., Greig(1) and Furchgott et al.(2). They interpreted then the changes observed under anoxia *in vitro* as indicative of what ought to have happened in shock.

This report describes tests on the Pasteur

reaction with brain tissue of rats, obtained during shock.

Methods. The shock procedure has already been described(3). Only "taped" rats were drummed. Their weights ranged from 150 to 250 g. The brains were excised and homogenized in Potter-Elvehjem homogenizers(4) with Teflon pestles under ice cooling in Krebs-Ringer or bicarbonate media(5) for aerobic and anaerobic tests, respectively. Each gram of fresh brain was made up with 10 ml of medium. The nitrogen content of the homogenates was determined with a mi-

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TABLE I. Pasteur Reaction in Rat Brain Homogenates.

	No.	Ratio of aerobic/anaerobic glycolysis	S.E.
Control	7	.17	.031
Trauma	7	.29	.037

"Student's" $t = 2.5$ $p < .05$.

Difference between control and trauma groups is statistically significant.

cro-Kjeldahl method. Non-protein nitrogen was measured after precipitation of an aliquot with 5% trichloroacetic acid (1:10) in the supernate and the difference was taken as protein nitrogen.

Aerobic glycolysis. 1.0 ml of brain suspension was pipetted into 25 ml Erlenmeyer flasks containing 1.0 ml of 10% glucose and 3.0 ml of a mixture of .85% sodium chloride and .05% potassium chloride of pH 7.4. For blank tests glucose was omitted and 4.0 ml of the salt mixture were used. The flasks were shaken in the Dubnoff shaker for one hour. Aliquots of 1.0 ml were then withdrawn and pipetted into 9.0 ml of 5% trichloroacetic acid for deproteinization. After centrifuging lactic acid was determined with the method of Barker and Summerson(6).

Anaerobic glycolysis. Homogenates were gassed for 30 minutes with a mixture of 95% nitrogen and 5% carbon dioxide in Warburg vessels. Ten per cent glucose and sodium chloride-potassium chloride mixture respectively were placed in the sidearms and tipped into the main compartment after thermal equilibrium had been reached. Analyses were done as described above.

All incubations were done at 37°C. Units of activity were micrograms of lactic acid formed from glucose/hr/mg protein nitrogen.

Glycogen. The anthrone method of Carroll *et al.* was used(7). Between 85 and 95%

of glycogen (Pfanstiehl) added to brain tissue was recovered by this method. The recovery from liver and muscle was 92 to 95%.

Results. Both aerobic and anaerobic glycolysis were increased in shock, 65% and 3% respectively (Table I). The ratios of aerobic and anaerobic glycolysis calculated for control and for shock provided a measure of comparison. These ratios were 1:4.96 for control and 1:3.10 for shock with a statistical significance of $P < .05$. This is, then, an increase of lactic acid formation from glucose in presence of oxygen or a repression of the Pasteur reaction in shock. The Pasteur reaction was 1.6 times less in shock than in the control. The change of glycogen content in shock is shown in Table II. Glycogen content of liver and muscle was likewise found to be decreased in shock.

Discussion. The homogenates from shocked rats continued to use the glycolytic pathway under aerobic conditions with a correspondingly decreased yield of ATP. Our earlier experiments(8) had shown that oxidative phosphorylation was uncoupled in mitochondria from shocked rats' hearts and that the phosphorylation of ADP was inhibited in brain preparations from shocked animals. It thus appears that the repression of the Pasteur effect and the uncoupling of oxidative phosphorylation went hand in hand in shock. Seits and Engelhardt(9) have demonstrated *in vitro* that the Pasteur reaction was decreased under the influence of poisons which caused uncoupling of oxidative phosphorylation. There are observations on 2 rat brains made by LePage(10) which show a sizeable decrease of glycogen content within 68 and 230 minutes respectively following 13 minutes of tumbling, when respiration had just ceased. These data bespeak the same trend observed by us and which we have

TABLE II. Glycogen Content of Rat Tissues, Control and Trauma, mg %.

	Brain			Liver			Muscle		
	No.	Avg contrast	S.E.	No.	Avg contrast	S.E.	No.	Avg contrast	S.E.
Control	22	48.56	2.28	5	3869.0	483.6	2	464.2	13.45
Trauma	22	42.95		5	1193.2		2	313.1	

"Student's" $t = 2.46$
 $P = .011$

"Student's" $t = 5.53$
 $P < .01$

"Student's" $t = 13.45$
 $P > .05$

shown to be significant. We observed a decrease of 20% only, whereas in the tests reported by LePage almost no glycogen was present. This difference may be explained by the fact that our samples were analyzed at a predetermined time when all rats were surviving and were not moribund.

The decrease of brain glycogen is in agreement with the impairment of the Pasteur reaction. Decreased phosphorylating processes in brain may contribute to the glycogen deficiency as was also shown for muscle and liver after shock. Low oxygen tension has been reported by Gurdijan *et al.* (11) to have decreased the glycogen content of the brain of one morphinized dog in severe hypoxia and cyanide poisoning causes also a highly significant decrease of glycogen in rat brain as seen by Albaum *et al.* (12). Burdette (13), as well as Kovach *et al.* (14) and Takacs *et al.* (15) have also found that in muscle there occurs depletion of glycogen in shock.

Summary. Increased aerobic and anaerobic glycolysis occurred in brains of tumbled (fettered) rats in shock. The Pasteur reaction was significantly decreased. This indicates an increased anaerobiosis in the brain tissue as a consequence of shock with concomitantly decreased yield of high energy phosphate. Glycogen content of brain is also

significantly decreased in shock.

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Immunologic Differences Between Murphy-Sturm Lymphosarcoma and Normal Rat Lymph Nodes.* (26272)

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The Murphy-Sturm lymphosarcoma of rats is derived from bloodborne leukemic cells (1), and is thus related to lymph tissue. This relationship was corroborated by the use of fluorescein label technics. We showed previously that rabbit antisera prepared against the ascites form of the lymphosarcoma strongly cross-react with rat lymph node and spleen (2).

We have now been able to show differences in the antigenic patterns of the lymphosarcoma and lymph node (taken as a normal counterpart) by the use of radio-iodine labeled antibody technics. I¹³¹ labeled globulins from anti-lymphosarcoma and anti-lymph node sera were specifically fractionated by successive adsorption and elution from tumor and lymph node components. It was thus possible to separate the antibodies into fractions of different specificities, indicating the presence of characteristic anti-

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genic components in each tissue. Previously, we had shown that antibodies against lymphosarcoma, either the solid tumor or ascites form, also contained antibodies directed against fibrin(2,3), but these appear to be different from the antibodies directed against the tumor cells themselves.

Experimental. Tumors: The Murphy-Sturm lymphosarcoma (ascites and solid form) has been described(4). Ascites lymphosarcoma cells (referred to as ALS) were collected from tumor-bearing rats 5-7 days after transplantation and washed with saline containing 0.5-1% sodium citrate at 5°C, then lyophilized.

Antisera. Anti-lymphosarcoma sera (anti-ALS) were prepared by immunizing rabbits with washed ascites cells in Freund's adjuvants as reported previously(2). Anti-rat lymph node sera (anti-LN) were obtained by injecting rabbits with lymph node homogenates mixed with Freund's adjuvants. The γ -globulin fractions of the sera were prepared by ethanol fractionation according to Deutsch (5). The globulin fractions were heated at 60°C for 30 minutes to remove thrombin activity before iodination. This was done in view of subsequent addition of whole plasma in some procedures. The procedures for iodination of proteins(6) and assay for radioiodine(4) have been described.

Preparation of sediments. Lyophilized ALS cells were suspended in borate-buffered saline containing 1% sodium citrate (pH 8, 5°C) and homogenized in a Potter-Elvehjem tissue grinder until no intact cells remained. The sediments were separated by centrifuging at 25,000 RCF for 20 minutes at 5°C. These were washed several times in the same centrifugal fields in the cold, then lyophilized. Frozen rat lymph nodes were treated in the same way with buffered saline without sodium citrate. Sediments were used for adsorption after suspending in borate buffer at pH 8.0 by means of a tissue grinder.

Preparation of purified anti-ALS antibody (A_A) and anti-LN antibody (L_L): The radioiodinated globulin fraction of anti-ALS serum (40 mg) was treated with whole rat plasma (2 ml; antigen excess) to neutralize antifibrin antibodies. After incubation at

37° for 1 hr the mixture was allowed to stand overnight at 5° and centrifuged. The supernate was treated with ALS sediment (30 mg) at 37°C for 1 hr. The sediment was washed and the adsorbed antibodies were eluted at 60° in presence of added bovine serum albumin (10 mg, 5 ml borate buffer pH 8) as carrier to avoid surface denaturation. Then the eluate† was treated twice with 20 mg of finely dispersed rat fibrin to remove any trace of anti-fibrin activity (2% and 1% of the radioactivity was adsorbed). The resulting supernate is the purified anti-ALS antibody, (A_A). The purified anti-lymph node antibody (L_L) was similarly prepared from I^{131} labeled globulin fraction of anti-LN sera by adsorption and elution from LN-sediment.

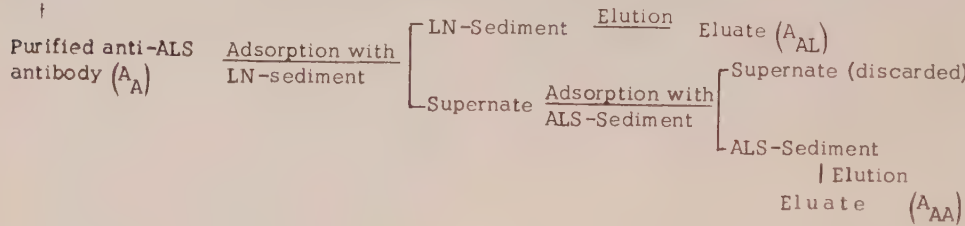
Fractionation of purified anti-ALS antibody (A_A): The scheme of the fractionation is provided in the footnote of Table I. Purified anti-ALS antibody (containing 184 μ g radioglobulin and 10 mg BSA) was treated successively with 25, 25 and 12 mg portions of lymph node sediment. 24, 8 and 4% of the original radioglobulin were adsorbed on the sediments. The sediments were pooled after thorough washing and eluted in borate buffer pH 8 containing 0.2% BSA. Fifty-one percent of adsorbed radioactivity was eluted. The eluate was referred to as A_{AL} . The supernate after treatment with lymph node sediments (90 μ g radioglobulin) was treated twice with ALS-sediment (30 and 15 mg) removing further 16 and 4% of the radioactivity (on the basis of purified antibody used) respectively. The sediments were combined

† It is known that anti-fibrin antibodies localize in the Murphy tumor when injected into tumor-bearing rats(3). Localizing activity of anti-ALS sera can be removed by treatment with fibrin. When the eluate prepared here was injected into tumor-bearing rats, localization in the tumor was found to be only about 1/10 of that of similarly prepared eluate without use of plasma. Further treatment with freshly formed rat fibrin clot completely removed the tumor localizing activity without affecting localization into normal tissues (e.g. liver). This indicates that only the fibrin-reacting antibody is contributing to the tumor localizing activity of anti-ALS sera and that the antibodies against cell components, dealt with in this report, do not localize in tumor *in vivo*.

TABLE I. Adsorption of Fractionated Anti-ALS Antibodies on ALS- or LN-Sediment.

Eluate used	1st adsorption*			2nd adsorption* (supernate of 1st adsorption)		
	Amt of I ¹³¹ globulin used, µg	Sediment used, mg	% adsorbed	Amt of I ¹³¹ globulin used, µg	Sediment used, mg	% adsorbed
A _{AA} †	4.2	ALS 7.5	40	.9	ALS 7.5	11
				.9	LN 6.3	7
	1.0	ALS 7.5	39			
	4.2	LN 6.3	19	1.4	ALS 7.5	22
A _{AL} †	1.0	LN 6.3	21	1.4	LN 6.3	9
	4.2	ALS 7.5	58			
	1.0	ALS 7.5	57			
	4.2	LN 6.3	49			
	1.0	LN 6.3	54			

* Adsorption was carried out at 37°C, 1 hr, pH 8 in 0.1% BSA. Sediments were washed twice with 8 ml borate buffer and counted. Radioactivity on the sediment was expressed in terms of per cent of radioactivity present in original eluate. Portions of the supernate were treated with sediment as indicated under heading of second adsorption.



after washing and eluted (59% eluted). The eluate was referred to as A_{AA}.

Fractionation of purified anti-LN antibody (L_L): The scheme of the fractionation is provided in the footnote of Table II. Purified anti-LN antibody (containing 410 µg radioglobulin and 10% normal rabbit serum) was treated twice successively with 30 mg ALS-sediment each time. Twenty-one and 6% of the radioglobulin were adsorbed. The washed sediments were pooled and eluted (31% eluted). The eluate was referred to as L_{LA}. The supernate after treatment with ALS sediment (295 µg radioglobulin) was treated twice with LN-sediment (25 mg

each) removing further 19 and 5% of the radioactivity respectively. The washed sediments were combined and eluted (36% eluted). The eluate was referred to as L_{LL}.

Results and discussion. Purified anti-ALS antibodies (A_A) were first treated with LN sediment, then with ALS sediment and the adsorbed radioglobulin on each sediment was eluted to give 2 preparations A_{AL} and A_{AA} respectively.† These eluates were tested for their combining properties with either ALS or LN sediment and the results are shown in Table I.

The antibodies eluted from ALS sediment (A_{AA}) combined with ALS sediment to twice the extent as LN sediment indicating some additional components in the tumor sediment. A_{AA}, after being treated with ALS or LN sediment was further tested for completeness of adsorption by another treatment with ALS or LN sediment. It was found that the primary LN adsorption was not as effective as the ALS adsorption in removing anti-ALS antibody.

† In another series of experiments, the supernate after LN adsorption was divided and tested by adsorption with ALS, LN, spleen and liver sediments. ALS sediment adsorbed a much larger fraction of radioglobulin than did any of the other 3 sediments. Among the other 3, spleen and liver sediments adsorbed less than LN sediment suggesting that spleen and liver sediments contain less cross-reacting components to anti-ALS sera than LN sediment.

The adsorptions were carried out using different amounts of globulin (varied by a factor of 4) with the same amount of sediment. The fraction of radioactivity adsorbed was the same for both levels of globulin showing that the pickup was not a function of globulin-sediment ratio in this range.

The antibodies eluted from the lymph node sediment (A_{AL}) were adsorbed on LN and ALS sediments to similar extents, about 55%, indicating the presence of identical or cross-reacting constituents in these tissues.

These experiments show that the antibodies against insoluble components of ALS cells (other than fibrin) are of multiple nature: some are directed against common components of lymph nodes and ALS while others are directed preferentially toward components either unique to ALS or present at very low concentration in lymph node.

The antibodies adsorbed on sediment are directed against the insoluble components of ALS rather than against the soluble parts. This was shown by the fact that the soluble components of ALS do not interfere with adsorption of the antibody by ALS sediment.

In fact, when a preparation of purified anti-ALS antibody (1 μ g) was treated by 4 mg of ALS-sediment in presence of normal rabbit serum, 36% of the radioactivity was found on the washed sediment. Addition of the soluble fraction from ALS-homogenate (supernate after centrifugation at 78,400 RCF for 60 min; containing 4.2 mg protein) to the system did not significantly affect adsorption (34% adsorbed). The presence of common constituents in the soluble fraction and the sediment would cause such interference.

Similar experiments were carried out with anti-LN antibodies for comparison. Purified anti-LN antibodies (L_L) were first treated with ALS-sediment, then with LN-sediment and the adsorbed radioglobulin on each sediment was eluted to form L_{LA} and L_{LL} respectively. These eluates were adsorbed with both ALS and LN sediments to examine their specificities. The results (Table II) indicate that the antibodies against insoluble components of LN can be fractionated into those with 2 different specificities: one directed against common components of lymph node

TABLE II. Adsorption of Fractionated Anti-LN Antibodies on LN- or ALS-Sediments.

Eluate used	1st adsorption*			2nd adsorption* (supernate of 1st adsorption)		
	Amt of I^{131} globulin used, μ g	Sediment used, mg	% adsorbed	Amt of I^{131} globulin used, μ g	Sediment used, mg	% adsorbed
L_{LL} †	4.9	LN 6.3	63	1.0	LN 6.3	12
	1.0	LN 6.3	69	1.0	ALS 7.5	4
	4.9	ALS 7.5	11	2.0	LN 6.3	59
	1.0	ALS 7.5	14	2.0	ALS 7.5	9
L_{LA} †	5.2	LN 6.3	45			
	1.0	LN 6.3	48			
	5.2	ALS 7.5	46			
	1.0	ALS 7.5	48			

* Adsorption was carried out at 37°C, 1 hr, pH 8 in presence of 1 ml NRS. Sediments were washed twice with 8 ml borate buffer and counted. Radioactivity on the sediment was expressed in terms of percent of radioactivity present in original eluate. Portions of the supernate were treated with sediment as indicated under heading of second adsorption.

†

Purified anti-LN antibody (L_L)	Adsorption with ALS-sediment	ALS-Sediment	Elution	Eluate (L_{LA})
		Supernate	Adsorption with LN-sediment	Supernate (discarded) LN-Sediment Elution Eluate (L_{LL})

and ALS (L_{LA}), whereas the other directed preferentially toward components either unique to lymph node or present at very low concentration in ALS (L_{LL}).

Thus, the fractionation of antisera into fractions of different specificities indicates that lymphosarcoma and lymph node cells each contain a characteristic component(s) either unique to itself or present at very low concentration in the other. The lymph node is taken as the most closely related normal tissue to lymphosarcoma. However, interpretation of the above data is limited to the difference between these tissues. Whether this tumor contains a unique substance or a substance present only in a particular minor cell line in the normal lymph node remains to be seen. The question of the correct normal cells with which to compare tumor cells is an underlying problem in cancer chemistry.

Summary. Rabbit antisera prepared against rat Murphy-Sturm lymphosarcoma (ascites form) and normal rat lymph node exhibit extensive cross reactions with these tissues. However, antibodies in the antisera were separated into fractions of different spe-

cificities: some directed against certain characteristic antigen(s) in the tumor or in the lymph node sediment and others against the common (cross-reacting) components in both. This was done by successive adsorptions of the antisera with the tumor and lymph node sediments and elution of adsorbed antibodies from these sediments. I^{131} labeled antibody was used for the studies.

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Latex Agglutination by the Reaction of Human Growth Hormone with Its Antiserum.* (26273)

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The specific antigenicity of human growth hormone has been demonstrated (2,3,4). The present study involves a reaction of human growth hormone with its antiserum and utilizes a simple agglutination technic. The purpose of this article is to report this technic.

Method. The method of antiserum production was a modification of the method outlined by Cohn (5). One mg of human growth hormone[†] made according to the Raben tech-

nic (1) was dissolved in one ml of water which had been adjusted to pH 3.0 with HCl. To this solution was added one mg of heat killed *Mycobacteria tuberculosis* and one ml of a mixture containing 9 parts of Bayol F (Penola Oil Co.) and one part of Arlacel C (Atlas Powder Co.). This mixture was emulsified with the growth hormone solution and injected into the toe pads of a young adult albino rabbit. Three weeks later the same amount of emulsion was prepared without the killed *Mycobacteria tuberculosis* and one-half injected in each of 2 sites intramuscularly. Three weeks later the rabbit blood was obtained by cardiac puncture and the serum

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† Kindly supplied by Dr. M. S. Raben.



FIG. 1. Latex agglutination by reaction of human growth hormone with its antiserum. Upper row: Rabbit antiserum. Lower row: Control rabbit serum. Both rows: From left to right, tubes 1 through 6, progressive dilutions of human growth hormone 2.8 to 0.086 μg ; tube 7, control. Tubes 1 through 4, positive in upper row.

was separated. Globulins were then precipitated from the antiserum according to the method of Deutsch(6) for rabbit serum. This precipitate, labeled Precipitate A, contained all of the serum globulins. A 0.5% stock solution of the lyophilized globulin precipitate was made in .06M Sorensen's phosphate buffer, pH 7.4. A latex-globulin suspension was then prepared by mixing 0.5 cc of 0.5% stock globulin solution, 0.1 ml of Difco Bacto-latex (particle size 0.81 μ) and 9.4 ml of the same phosphate buffer. Control rabbit blood was treated similarly.

Growth hormone(1) 0.5 mg was dissolved in 1.0 ml of water acidified to a pH of 3.0 with HCl. The growth hormone solution was then diluted with 0.06 M Sorensen's phosphate buffer, pH 7.4, to the desired dilution. Serial dilutions of growth hormone with a volume of 1.0 ml were then made with the phosphate buffer in the first 9 of a set of 10 tubes. The tenth tube contained only one ml of buffer. To each of these tubes was added one ml of the latex-globulin suspension. The tubes were then shaken. A similar set of tubes using control globulin was set up. The tubes were heated for 30 minutes at 37°C, then allowed to sit at 4°C for 120 minutes. After warming to room tem-

perature they were incubated at 56°C for 90 minutes; this increased the agglutinating properties of some antisera. They were then allowed to sit at room temperature overnight at which time results were read visually.

Results. Fig. 1 illustrates the agglutination, and Table I shows the reaction quantitatively. This table also shows that agglutination occurs over a restricted range of antiserum/growth hormone concentration and does not occur when antigen concentration is excessive or too little. Table II shows that latex agglutination inhibition occurs if the growth hormone is mixed with the antiserum before a latex-growth hormone suspension is added. This is accomplished by first adding antiserum to progressive serial dilutions of growth hormone and adjusting the volume to one ml and with phosphate buffer. After incubation at 37°C for 30 minutes and sitting at 4°C for 30 to 120 minutes one ml of a latex-growth hormone suspension containing 2.0 μg of growth hormone, 0.1 ml of Difco Bacto-Latex, and 9.9 ml of phosphate buffer is added. The procedure from this point is as described in "Methods" above after addition of the latex suspension.

Discussion. Hayashida and Li(2) showed that human growth hormone antiserum would neutralize the biologic potency of growth hormone; they were also able to produce guinea pig anaphylaxis by the reaction of growth hormone with its antiserum. The results of the experiments of Grumbach *et al.*(4) using precipitating technics give strong evidence

TABLE I. Latex Agglutination* by Immunologic Reaction of Human Growth Hormone with Rabbit Serum Globulin.

Tube No.	Human growth hormone (μg)	Anti-serum globulin	Control serum globulin
1	46.0	0	0
2	23.0	0	0
3	11.5	+	0
4	5.7	++	0
5	2.8	++++	0
6	1.4	++++	0
7	.7	++	0
8	.3	+	0
9	.1	0	0
10	0	0	0

* Degree of agglutination is expressed by No. of plus marks. A negative reaction is expressed by 0.

TABLE II. Latex Agglutination* Inhibition Test by Immunologic Reaction of Human Growth Hormone with Rabbit Serum.†

Tube No.	Human growth hormone (μ g)	Row I, antiserum	Row II, control serum
1	3.10	0	0
2	2.66	0	0
3	2.28	0	0
4	1.90	0	0
5	1.52	0	0
6	1.14	+	0
7	.76	+	0
8	.38	+	0
9	.19	++	0
10	.15	++	0
11	.11	++	0
12	.08	++	0
13	.04	+++	0
14	.02	++++	0
15	.01	++++	0
16	.00	0	0

* Degree of agglutination is expressed by No. of plus signs. No reaction is expressed by 0 sign.

† .005 ml of rabbit serum was used in each tube except for tube 16 in each row.

that there is one antigen-antibody reaction in the reaction between the Raben growth hormone product and its antiserum. Read(3) using rabbit antisera to the same product was able to show by a hemagglutination-inhibition technic the presence of an antigen in increased amounts in sera of acromegalic patients and decreased amounts in patients with hypopituitarism when compared to normal individuals. Assuming, then, on the basis of this evidence, that human growth hormone is antigenic, we conclude that latex agglutination as demonstrated by the results of this

experiment is a manifestation of the reaction of human growth hormone with its antiserum.

The results in Table I show a zone of optimal concentration for antigen and antibody as manifested by a zone of no agglutination in the region of antigen excess and a zone of no agglutination where antigen concentration is too little. This follows well-known laws of other antigen-antibody systems using rabbit antisera(7).

It is hoped that latex agglutination by the reaction of human growth hormone with its antiserum may be useful for detection of growth hormone in human biological fluids. Experiments are now in progress to test this possibility.

Summary. A method has been described which demonstrated that the reaction between human growth hormone and its antiserum caused agglutination of a suspension of latex.

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Ammonia Detoxication in Liver from Humans.* (26274)

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The recent development of methods for assaying the individual enzymes of the urea cycle in a single homogenate(1) have made possible accumulation of data on ammonia

detoxication by this enzymatic mechanism in human liver. With similar data for glutamic acid dehydrogenase and glutamine synthetase, it is possible to calculate the amount of ammonia which *theoretically* could be fixed by these separate mechanisms, and to obtain an over-all estimate for *in vitro* fixation of

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ammonia by this tissue. Development of the enzymes for urea synthesis in fetal liver has also been studied. Previous studies have shown that there is an increase in human fetal liver arginase after the fourth month(2). Urea formation has also been demonstrated in human fetal liver at this stage with tissue slices and the reaction mixture described by Krebs and Henseleit(3). Glutamine synthetase and glutamic acid dehydrogenase activities have, to our knowledge, not been studied in human liver.

Materials and methods. A single specimen of fresh liver was obtained at 12, 20 and 40 weeks of gestation and from 2 adults during surgery.† Activities of the urea cycle enzymes were determined as previously described(1). Glutamic acid dehydrogenase was determined spectrophotometrically by following the decrease in optical density as a result of oxidation of DPNH during the course of reductive amination of α -ketoglutarate to glutamate. The reaction mixture was essentially that described by Solomon(4) and contained: 1 μ mole of DPNH; 250 μ moles of phosphate buffer (pH 7.4); 300 μ moles of NH_4Cl ; and 0.1 ml of appropriately diluted enzyme (1 part liver plus 99 parts of double distilled water) from a distilled water homogenate in each cuvette. Final total volume was 3 ml. After setting the spectrophotometer for the blank (all of the reagents except the DPNH), 20 μ moles of α -ketoglutarate were added to the other cuvette at 0 time and mixed rapidly with a stirrer. The decrease in optical density at room temperature was then recorded in a Beckman DUR spectrophotometer. One unit of glutamic acid dehydrogenase activity is defined as that amount which will oxidize 1 μ mole of DPNH in one minute of the initial reaction. The value of the molecular extinction coefficient of DPNH employed was that determined by Horecker and Kornberg(5). Glutamine synthetase was determined by measurement of glutamyl hydroxamate formation according to the method of Lipmann and Tuttle(6). The reaction mixture con-

TABLE I. Urea Cycle Enzymes in Human Liver.

		Prenatal (wk gestation)			Adult (yr)	
		12	20	40	18	46
Carbamyl phosphate synthetase						
U	91	95	121	256	222	
SA	1.4	1.3	3.8	3.1	3.1	
Ornithine transcarbamylase						
U	599	1461	4234	9000	7910	
SA	9.1	20	132	112	110	
Arginine synthetase*						
U	13	18	21	55	48	
SA	0.29	0.23	0.52	0.78	0.77	
Cleavage enzyme						
U				168	180	
SA				2.4	2.9	
Arginase						
U	2842	5054	5107		7750	
SA	43	70	160		108	
Estimated urea synthesis, g (see text)						
	.021	.321	3.19		101 (avg)	

The data of Jackson(8) were used for liver weights at 12 and 20 wk. Liver of 40 wk fetus weighed 105 g; 1400 g was used as wt for liver of adult female. Definition of U (units) and SA (specific activity) is given in the text under *Results*.

* Arginine synthetase represents activities of both condensing and cleavage enzymes(1).

tained: 200 μ moles of hydroxylamine hydrochloride (neutralized with KOH); 10 μ moles of ATP (di-K salt); 50 μ moles of K-glutamate; 260 μ moles of tris (trihydroxymethylaminomethane) buffer (pH 7.5); 20 μ moles MgCl_2 ; and 0.5 ml of homogenate (1 vol. of liver in 4 vol. of 0.15M KCl) in a final volume of 2.3 ml. The reaction was carried out at 38° for 15 minutes and deproteinized with 0.4 ml of 40% trichloroacetic acid. The ferric chloride reagent was then added and after centrifugation an aliquot of 1 ml was read at 500 m μ in the Beckman DU spectrophotometer. A standard curve was prepared using succinyl hydroxamate from which the value for the glutamyl derivative was approximated from the data of Meister *et al.*(7).

Results. The values obtained for the urea cycle enzymes in units and specific activity are given in Table I. The units represent μ moles of product formed per gram of wet tissue per hour and specific activity represents μ moles of product produced per mg of protein per hour. If a calculation is made

† Age, 46; gastric ulcer; age 18; asymptomatic gall stones. No clinical evidence of liver disease.

TABLE II. Enzyme Systems Fixing Ammonia.*

Liver tissue	Glutamic acid dehydrogenase†				Glutamine synthetase†		
	μ moles DPNH oxidized/min.		Units/g tissue	Units/mg prot.	μ moles glutamyl hydroxamate		μ moles glutamine, g prot./15 min.
	Per ml	In cuvette			React. mix	Per g tissue	
Human	5.64×10^{-3}	16.9×10^{-3}	169	0.39	0.8	1.6	15.7
Rat	7.8×10^{-3}	23.4×10^{-3}	234	1.33	1.75	3.5	156.

* Urea cycle enzymes are given in Table I.

† Assay conditions as described in text.

for amount of urea which could be formed *in vitro* in a 24 hour period from the rate limiting step (arginine synthetase system), one obtains the values appearing in the bottom line of the table. If calculations are made for $\text{NH}_3\text{-N}$ which could be incorporated directly into glutamic acid and glutamine in adult liver, the following values are obtained for a 1 hour period: 40 g of $\text{NH}_3\text{-N}$ for glutamic acid and 627 mg for glutamine (Table II). These are to be compared with values of 22.1 mg of $\text{NH}_3\text{-N}$ as glutamic acid and zero as glutamine in fetal liver (20 wks). Since only half the nitrogen of urea comes from free ammonia, it is calculated that 23.6 g of $\text{NH}_3\text{-N}$ could be fixed in a 24 hour period by this mechanism. Glutamic acid dehydrogenase would thus seem to have the greatest *potential ability* for ammonia detoxication.

Discussion. Duda and Handler(9) have shown by injection of N^{15}H_3 into rats that free ammonia is removed primarily by formation of glutamine and urea. In their studies, 65% of the administered isotope was present in glutamine and urea at the 15 minute interval and 90% in 30 minutes (72% as glutamine and 18% as urea). They have also made a calculation for glutamine turnover in the rat which suggests that 80% of NH_3 freed by the transamination and deamination reactions of glutamine is resynthesized into glutamine. The NH_3 lost during this turnover is of the same order of magnitude as the direct incorporation of NH_3 into urea during this period. Maximal rate of urea synthesis in the rat given an LD 99.9 dose of ammonia, also calculated by Duda and Handler(9), was about 1140 mg of urea for a 300 g rat. This can be compared with the figure given for urinary excretion of urea (720 mg) in the

albino rat for the same period(10). Calculations of data from enzyme studies on urea formation in homogenates of rat liver indicate that the adult rat with a liver of 12 g(11) can synthesize of the order of 710 mg of urea in a day. These figures are all very close and suggest that the rat has very little *reserve synthetic ability* in the liver for urea synthesis.

In human beings, the enzymatic data suggest that only one-fifth of *potential synthetic ability* of the liver is used for daily synthesis of urea. In comparing data for adult human liver with that for the rat, one finds that, on the assumption that 20% of $\text{NH}_3\text{-N}$ fixed as a result of glutamine synthesis is eventually converted to urea, the human could synthesize about 12.9 g of urea per day. Since this value is lower than the average daily excretion of the adult human and would be smaller by about 20% because of degradation in the intestinal tract(12), it is obvious that free ammonia, other than that arising from glutamine, is a major source for urea synthesis. In the case of fetal liver, glutamine cannot be a source of ammonia for urea synthesis since there is no demonstrable glutamine synthetase activity. This observation is consistent with the enzymatic findings that glutamine is not directly involved in urea biosynthesis.

The quantitative importance of glutamic acid dehydrogenase in ammonia detoxication in the liver is far from clear. Its role may be more important in the brain and in fetal tissues(4).

The greater capacity to synthesize arginine (by way of the ornithine-urea cycle) in the case of the human as compared with the rat could explain the need for arginine for maximum growth of the rat.

During human pregnancy, the urea formed

by the fetus is allegedly excreted by the mother. Balance studies show a positive nitrogen balance during pregnancy(13). From the present data, it is seen that the human fetus could produce as much as 3 g of urea daily. This amount of nitrogen would be within the normal daily variation of nitrogen excretion of the mother and thus nitrogen balance studies would not reflect the contribution of the fetus to maternal nitrogen excretion.

Summary. The detoxication of ammonia in homogenates of liver from fetal and adult human beings has been studied. Amount of urea synthesized in a day for each case has been calculated from the rate-limiting step of the urea cycle. Similar data are presented for glutamine synthetase and glutamic acid dehydrogenase. Values for human beings have been compared to similar values for the rat, and differences have been discussed.

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Experimental Steatorrhea Induced in Man by Bile Acid Sequestrant.* (26275)

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Bile acids are believed necessary for efficient digestion and absorption of dietary fat. An agent capable of binding bile acids in the gut might be expected to cause steatorrhea. In the present study, steatorrhea was induced experimentally in a series of otherwise healthy human subjects by means of a bile acid sequestrant (MK-135). This agent is the chloride salt of a basic anion exchange resin which binds bile acids *in vitro* and which, when administered orally, forms an insoluble, nonabsorbable complex with

bile acids in the intestinal lumen(1,2).

Methods. Sixteen healthy adults aged 24-32 served as subjects. Effect of MK-135[‡] administration on fecal fat excretion was determined in 4 subjects, and influence of this agent upon absorption and fecal excretion of I¹³¹-labeled triolein or I¹³¹-labeled oleic acid was measured in 12 subjects. **Fat balance studies.** To insure constant intake, 4 subjects were maintained on formula-type diets providing 40 calories/kg body weight/day for 5-6 weeks. Formula derived 40% of calories from fat (corn oil in two subjects, butter in one, and coconut oil in one), 43% from carbohydrate (dextrose) and 17% from

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[†] Postdoctoral fellow of Nat. Heart Inst., U.S.P.H.S.

[‡] Kindly supplied by Dr. Edmund DeMaar, Merck Sharp & Dohme Research Laboratories, West Point, Pa.

TABLE I. Effect of Administration of Bile Acid Sequestrant (MK-135) on Daily Mean Fecal Fat Excretion (g/Day) in 4 Subjects Maintained on Constant Formula-Type Diets.

Subject	Dose of MK-135, g/day*	Period I Control (10-16 days)	Period II Experimental (11-18 days)	Period III Control (10-16 days)
1	15	1.1 $\pm$.3†	2.2 $\pm$.9	1.0 $\pm$.5
2	15	.6 $\pm$.1	1.0 $\pm$.4	1.0 $\pm$ 1.3
3	30	3.0 $\pm$.8	12.7 $\pm$ 1.7	2.9 $\pm$.9
4	30	2.0 $\pm$.4	10.2 $\pm$ 1.1	2.0 $\pm$.3

* Given during experimental period only.

† Stand. error.

TABLE II. Mean Values for Blood and Fecal Radioactivity Following Ingestion of I¹³¹-Labeled Triolein (5 Subjects); and I¹³¹-Oleic Acid (7 Subjects), before and during Administration of Bile Acid Sequestrant (MK-135).

	% of dose/liter of blood				% of dose in feces (48hr)
	2 hr	4 hr	6 hr	8 hr	
Subjects (5) given I ¹³¹ -triolein					
Control	.5 $\pm$.3*	2.1 $\pm$.2	2.5 $\pm$.6	1.9 $\pm$.4	2.1 $\pm$.8
During MK-135, 30 g/day	.3 $\pm$.1	.6 $\pm$.1	.6 $\pm$.1	.6 $\pm$.1	21.5 $\pm$ 6.1
Subjects (7) given I ¹³¹ -oleic acid					
Control	1.2 $\pm$.3	2.1 $\pm$.1	1.9 $\pm$.1	1.7 $\pm$.3	6.2 $\pm$ 1.5
During MK-135, 30 g/day	1.4 $\pm$.3	2.1 $\pm$.2	1.8 $\pm$.1	1.4 $\pm$.1	7.6 $\pm$ 1.1

* Stand. error.

p < .01.

protein (Casec⁶). During experimental periods lasting 11-18 days, MK-135 was administered 4 times daily as liquid suspension (0.25 g resin/ml). Two subjects received 15 g and two, 30 g of resin/day. Each experimental period was preceded and followed by control periods lasting 10-16 days. Feces were collected in 24-hour lots and aliquot samples analyzed for total fat content by method of Van de Kamer *et al.* (3). *Fat absorption studies.* Five subjects were given a standard dose of I¹³¹-labeled triolein. Radioactivity was measured over an 8-hour period in blood samples and over a 48-hour period in feces. Subjects were then given 30 g/day of resin in divided doses for one week while on regular diet. On last day of resin administration, I¹³¹-triolein test was repeated. Similar studies were done in 7 additional subjects except that a standard dose of I¹³¹-labeled oleic acid was administered during control and experimental periods. All subjects were studied while in postabsorptive state, and had received 0.5 ml Lugol's solution 14 hours before each administration of radio-

active material. First meal of day was allowed not earlier than 4 hours after ingestion of radioactive material.

Results. In 2 subjects maintained on constant dietary intake and receiving 15 g/day of resin, there was no significant difference in daily fecal excretion of fat when experimental periods were compared with control periods. In contrast, steatorrhea occurred in 2 subjects receiving 30 g/day of resin (Table I). The gross character of the steatorrhea was evident by inspection as well as by chemical analysis of feces. All subjects noted borborygmi, increased frequency and greater bulk of stools during the experimental period. In 5 subjects maintained on regular diet and given I¹³¹-labeled triolein before and during 30 g/day resin treatment, there was depression of level of blood radioactivity over the 8-hour sampling period, and significant increase in fecal radioactivity during period of resin administration (p < 0.01) (Table II). In contrast, in 7 subjects maintained on regular diet and given I¹³¹-labeled oleic acid, there was no significant difference in radioactivity of blood and feces between control and experimental periods (Table II).

⁶ A fat-poor form of casein kindly supplied by Mead-Johnson and Co., Evansville, Ind.

Discussion. Gross steatorrhea in otherwise healthy subjects was induced by administration of bile acid sequestrant in appropriate dosage. Such steatorrhea was associated with deficient digestion and absorption of ingested triglyceride as shown by fat balance studies and I^{131} -triolein studies. In contrast, there was no interference with absorption of I^{131} -oleic acid during period of resin administration. These studies suggest that the mechanism of resin-induced steatorrhea is related to exclusion of bile acids from participation in hydrolytic digestion of dietary triglyceride.

Malabsorption has been reported following administration of neomycin(4), and trimethyl hexadecyl ammonium stearate(5). In the former type large doses of neomycin were required and steatorrhea was inconsistent. In the latter, chemical used was very toxic to animals, producing moderate to severe damage of intestinal villi. In contrast, use of MK-135 appears to be an innocuous and ef-

fective method for induction of steatorrhea in man.

Summary. Gross steatorrhea has been induced experimentally in healthy human subjects by administration of a resin capable of sequestering bile acids in the intestinal lumen. This agent (MK-135) inhibited absorption of I^{131} -labeled triolein but not I^{131} -labeled oleic acid. In contrast to certain other forms of experimental malabsorption, MK-135-induced steatorrhea appears to be predictable and innocuous.

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Antiphage Activity of Human Cholera Sera. (26276)

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Although Doorenbos and Cossery(1) have reported on the inactivation of cholera phage by rabbit antisera, a search in pertinent literature failed to find evidence of studies concerned with the anticholera phage activity of human sera. It was believed, therefore, that such an investigation of the sera from cholera patients might yield significant results.

Materials and procedures. One hundred seventy-seven sera collected during the 1958-1960 cholera epidemic in Thailand and preserved in the frozen state at -20°C were used. There were 37 pairs of sera from cholera patients. One sample was drawn during the first or second day of the disease, while the other was collected 12 to 24 days later.

Eighteen paired sera were obtained from symptomless carriers of *V. comma*; 20 from contacts who had no clinical signs of cholera and from whose stools *V. comma* was not isolated; and 30 from patients with acute shigellosis or salmonellosis. All were collected in 12 to 25 day intervals. Thirty-five unpaired sera were from apparently healthy soldiers of the Royal Thai Army. Approximately 90% of all sera, including those from bacteriologically proven cases of cholera, came from individuals who were recently vaccinated against cholera.

A bacteriophage, isolated from a canal in Bangkok, which belonged to the first group according to the classification of Mukerjee

et al.(2) was employed, together with a *V. comma* Ogawa strain cultured from a classic case of cholera in Bangkok which was classified by Dr. Mukerjee as phage type 1.

The propagation of the phage and of the Ogawa organism was carried out according to Mukerjee *et al.*(2), while evaluation of the inhibitory power of the sera was undertaken by closely following the method of Toussaint and Muschel(3). The phage was used in a dilution of 10^{-3} . Two-fold serial dilutions of the sera, from 1:10 to 1:320, were employed. The phage-serum mixtures were incubated for 2 hours at 37°C before addition to the vibrio strain. The highest dilution of sera causing at least 50% inactivation was considered the titer. Paired sera from the same individual were tested on the same day.

Results. All tested sera at 1:10 dilution produced at least a 50% inhibition of the activity of the diluted cholera phage. Those collected during the acute cholera, from carriers, contacts, noncholeraic diarrhea and from symptomless soldiers never showed such activity in dilutions greater than 1:80. The second sera from carriers, contacts and noncholeraic diarrhea either inactivated cholera phage in the same dilution as the first sera (49 out of 68) or in the next higher (9 out of 68) or the next lower (6 out of 68) dilution. Only 4 of the second sera in this group showed a greater increase or decrease in the titer. On the other hand, sera drawn 12 to 24 days after onset of clinical cholera demonstrated an antiphage activity in at least 1:20 and in dilutions as high as 1:320. Average increase in the titer of the convalescent over the acute specimens was approximately 3-fold. The results in patients with cholera are demonstrated in Table I.

Discussion. Inhibition of phage activity

by normal sera had been demonstrated by Toussaint and Muschel(3) who used bacteriophage of the *E. coli* T series. The work reported here indicates that a cholera phage of type I is also susceptible to inactivation by sera. It is possible that human sera in low dilutions will inactivate many of the phages which lyse enteric bacteria.

A significantly greater antiphage activity was observed after convalescence from cholera and may be related to increased levels of phage antibody.

It is well known that cholera phage is excreted at the end rather than at the beginning of cholera(4). Thus the increase in antiphage activity after illness is not surprising.

Keller and Engley(5) and Keller and Zatzman(6) showed that phage disappears from the bodies of experimental animals quite rapidly. It is not known, however, if cholera phages ever enter the human circulation. Thus one can only conjecture whether the classical antibody-complement system or other serum components are involved in anticholera-phage activity. One has to note, however, that an increase in antiphage activity occurred only in the sera of those individuals with clinical disease and not during the carrier status nor in exposed persons who undoubtedly ingested *V. comma* at one time or another. The increased antiphage activity is thus closely associated with clinically manifest cholera. Obviously further studies of phage neutralizing activity of body fluids in health and sickness are required in cholera and in other diseases.

Summary. Increased anticholeraphage activity of human sera was observed after cholera but not in carriers, contacts and healthy controls.

The author is indebted to Major Louis H. Muschel, Walter Reed Army Inst. of Research, Washington, D. C., and to Dr. S. Mukerjee, Indian Inst. for Biochemistry and Exp. Med., Calcutta, for most helpful suggestions.

TABLE I. Anticholeraphage Titers of Sera from Cholera Patients.

No. of patients	Reciprocal titer during acute illness	Reciprocal titer after convalescence in No. of patients				
		20	40	80	160	320
23	20	2	4	12	2	3
12	40	1	1	2	5	3
4	80	0	1	1	0	2

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Cholesterol-4-C¹⁴ and Bile Acids in the Guinea Pig.* (26277)

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Cholic acid (3 α , 7 α , 12 α -trihydroxycholan-ic acid) has been isolated from the bile of many animal species. Because of the reported absence of 3 α , 7 α , 12 α -trihydroxycholan-ic acid in the bile, the guinea pig has been termed "unique"(1). Recently we have found that 3 α , 7 α , 12 α -trihydroxycholan-ic acid is the most abundant bile acid in the adult guinea pig, although absent in the immature animal(2). In determining the bile acid pattern of a particular species, several aspects have emerged: a) conversion of sterols into several bile acids having species-variable interconversion(1), b) oxidation of side chain in relation to 12-hydroxylation, and c) modification of hydroxyl groups during enterohepatic cycling(3). In studies on formation of bile acids the bile has been obtained from gall bladders and from biliary tract fistulae. In preliminary studies we found that the voluminous flow of bile from biliary fistulae resulted in early death of the guinea pigs. Recycling of bile or replacement of bile removed from the catheterized biliary tract permitted long term survival of the guinea pigs and the present study of conversion of cholesterol-4-C¹⁴ into labeled bile acids under favorable physiological conditions and at different phases of the enterohepatic cycling.

Methods. Young adult guinea pigs of either sex, approximately 5 months of age and weighing 500-700 g, were used in this study. A bile fistula was performed as described previously(2) in all animals. Forty-eight hours after operation, cholesterol-4-C¹⁴

was injected into the ear vein. For study of continuous enterohepatic cycling the catheters remained connected and small samples of bile were removed periodically. For study of freely flowing bile the 2 catheters were not connected until the ninth hour after administration of cholesterol-4-C¹⁴. Aliquots of the samples of the free-flowing bile collected from 0-1, 1-3, and 3-6 hours after injection were given to different guinea pigs intraduodenally *via* common bile duct catheter (Fig. 1). The free-flowing bile from these animals was then collected for 9 hours. In all studies of free biliary flow a volume of normal unlabeled bile equal to that diverted was supplied through the duodenal fistulae. After 9 hours of free flow the catheters were connected and thereafter small daily samples of bile were taken in all animals.

For the continuous enterohepatic cycling 0.450 μ C (29.5 μ g), and for 9 hours free flow 0.900 μ C (59 μ g) were used. Radioactivity in the bile samples infused intraduodenally into other guinea pigs was: 0-1 hr, 0.0651 μ C; 1-3 hr, 0.0671 μ C; 3-6 hr, 0.0334 μ C.

Bile acids were separated and identified by procedures used previously(2). After removal of the conjugated bile acids from the columns, residual radioactive materials ("neutral sterols") were eluted with chloroform. Aliquots of the initial bile samples, extracts and fractions of column effluents were plated on aluminum planchets and C¹⁴ determined with a windowless flow counter with sufficient accumulated counts per sample for 1% standard error. The paper chromatograms were scanned for radioactivity

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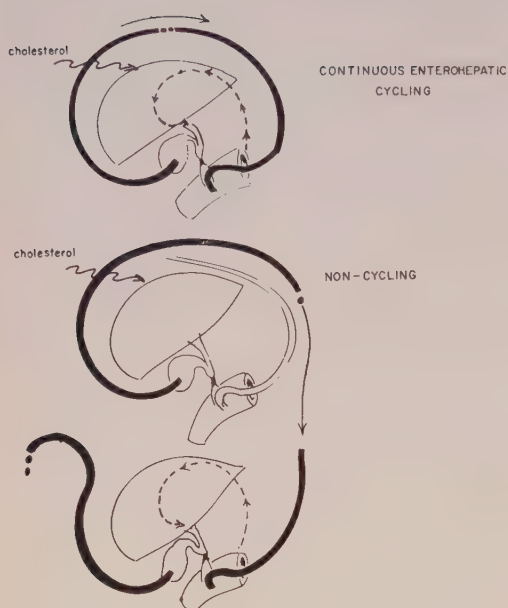


FIG. 1. Gall bladder and common duct catheterization with ligated common bile duct for determination of effects of continuous recycling, $\frac{1}{2}$ cycle or tissue to bile outflow, and single enterohepatic cycle.

with a gas flow window counter. Those samples containing insufficient radioactivity for individual processing were pooled.

Results. After intravenous injection of cholesterol-4-C¹⁴ in the guinea pig, 3 bile

acids were isolated from all bile samples: 3 α , 7 α - dihydroxycholanolic (chenodeoxycholic), 3 α - hydroxy, 7 - ketocholanolic (7 - ketolithocholic) and 3 α , 7 α , 12 α -trihydroxycholanolic (cholic) acid. Of these bile acids, 3 α , 7 α -dihydroxycholanolic and 3 α -hydroxy, 7-ketocholanolic acid appeared more rapidly and in greater concentration (Fig. 2, 3, 4). Although 3 α , 7 α , 12 α -trihydroxycholanolic acid appeared more slowly in the bile, it gradually increased without attaining the relative quantity of the other 2 bile acids during the first 9 hours of either enterohepatic cycling or free flow from the fistulae. After one or more days of enterohepatic cycling 3 α , 7 α , 12 α -trihydroxycholanolic acid became the predominant labeled bile acid and maintained a high level for 2 weeks or more after the original injection of labeled cholesterol (Table I). Thus, formation of all 3 bile acids was independent of enteric passage or microorganisms, but the high level of 3 α , 7 α , 12 α -trihydroxycholanolic acid observed in the adult guinea pigs was found, under the conditions of the present experiment, only after enterohepatic cycling.

Cholesterol-4-C¹⁴ was converted rapidly into bile acids with 26.7% of the label being excreted as bile acids or "neutral sterols" during the first 9 hours of free biliary flow (Fig.

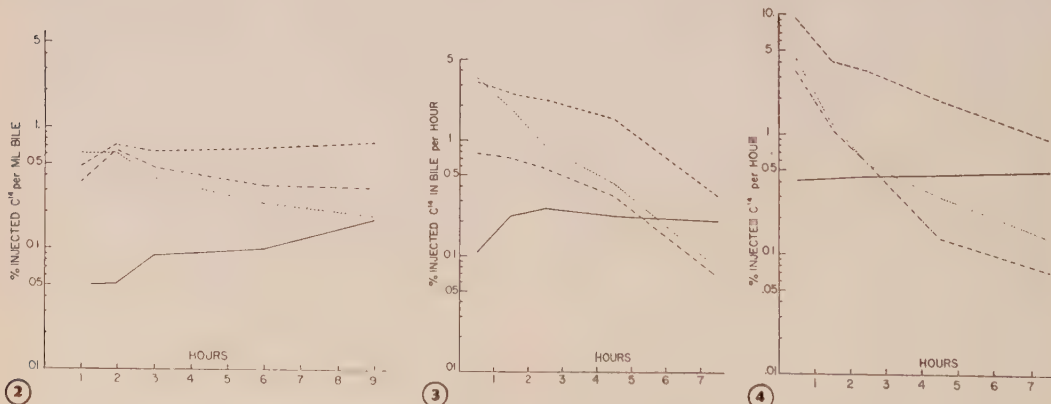


FIG. 2. Appearance of labeled bile acids and "neutral sterols" as % of inj. cholesterol-4-C¹⁴/ml in bile during first 9 hr of continuous enterohepatic cycling. — 3 α , 7 α , 12 α -trihydroxycholanolic acid. - - - - 3 α , 7 α -dihydroxycholanolic acid. ····· "neutral sterols."

FIG. 3. Labeled bile acids and "neutral sterols" in non-cycling (free-flow) bile for 9 hr after intrav. administration of cholesterol-4-C¹⁴. Explanation of graph as in Fig. 2.

FIG. 4. Biliary excretion of bile acids and "neutral sterols" for 9 hr after intraduodenal administration of labeled bile collected from another animal (0-1 hr sample of Fig. 3). Explanation of graph as in Fig. 2.

TABLE I. Labeled Compounds in Bile during Enterohepatic Cycling as % of Total Radioactivity in Sample and as % of Injected or Infused C¹⁴, Adjusted by Radioactivity Removed in Bile Samples: A, after intravenous injection of cholesterol-4-C¹⁴ and continuous cycling; B, after intravenous injection of cholesterol-4-C¹⁴ and free bile flow for 9 hr; C, D, and E, after intraduodenal infusion of labeled bile collected 0-1, 1-3 and 3-6 hr respectively.

Exp. condition	Time of enterohepatic cycling, hr	3 α , 7 α , 12 α -trihydroxycholanolic acid		3 α , 7 α -dihydroxycholanolic acid		3 α -hydroxy, 7-ketocholanolic acid		"Neutral sterols"	
		% C ¹⁴ in sample	% inj. dose/1.0 ml	% C ¹⁴ in sample	% inj. dose/1.0 ml	% C ¹⁴ in sample	% inj. dose/1.0 ml	% C ¹⁴ in sample	% inj. dose/1.0 ml
A	9- 24	19.0	.019	40.0	.041	24.0	.024	17.0	.017
	24-240	81.0	.037	12.5	.019	3.5	.005	3.0	.004
	240-432	92.8	.090	5.1	.004	.3	.0002	1.8	.0016
B	9- 24	33.1	.030	40.4	.037	10.0	.009	16.5	.013
	24- 96	51.0	.056	36.0	.038	5.6	.005	7.4	.008
C	9- 24	41.4	.199	40.0	.192	10.6	.050	8.0	.038
	24- 96	48.0	.135	39.0	.109	9.3	.026	3.7	.010
D	9- 24	45.6	.215	42.0	.198	7.3	.033	5.1	.023
	24-192	62.1	.214	27.0	.093	6.2	.020	4.7	.016
E	9- 48	50.0	.350	41.0	.288	7.7	.053	1.3	.008
	48-192	62.7	.392	30.3	.189	6.1	.037	.9	.005

3). The conjugated bile acids and "neutral sterols" were rapidly absorbed from the bowel, with 42.25%, 31.32% and 40.39% of the labeled substances being excreted in the bile during the first 9 hours after introduction of 0-1, 1-3 and 3-6 hour samples, respectively. The relative proportion of labeled bile acids infused intraduodenally was similar to that excreted in the bile during the first 9 hours, although a relatively greater amount of 3 α -hydroxy, 7-ketocholanolic acid than of the other 2 bile acids appeared initially. The proportion of labeled "neutral sterols" excreted to that infused was far less than that of the bile acids.

The major part of the labeled bile acids separated by column and paper chromatography was conjugated with taurine. However, during the first few hours after injection of cholesterol-4-C¹⁴, slight radioactivity was found in chromatographic regions of glycine conjugates. After separation of these fractions and after alkaline hydrolysis, the minute absolute amount of radioactivity prevented processing of different samples but chromatographic separation of pooled samples on column and paper revealed 2 labeled materials having the approximate mobility of 3 α , 7 α -dihydroxycholanolic and 3 α -hydroxy, 7-ketocholanolic acid. Although color tests with phosphomolybdic and phosphotungstic acid on paper chromatogram were negative and

no titratable material was present in the effluent from the column, the 2 labeled compounds cocrystallized with the respective unlabeled 3 α , 7 α -dihydroxycholanolic or 3 α -hydroxy, 7-ketocholanolic acid isolated from guinea pig bile and identified as previously reported(2). Furthermore, the C¹⁴/mg remained constant after several recrystallizations, as also observed with the free bile acids from taurine conjugates. The acetylated(4) bile acids also retained constant C¹⁴/mg. No C¹⁴ was found in the bile samples other than that present in the taurine or glycine conjugated bile acids and the "neutral sterols."

Discussion. This study on conversion of cholesterol-4-C¹⁴ into labeled bile acids confirms the previous report(2) on the predominance of 3 α , 7 α , 12 α -trihydroxycholanolic acid in the bile of the adult guinea pig. Although labeled 3 α , 7 α , 12 α -trihydroxycholanolic acid occurred independently of enteric passage, it appeared in low but gradually increasing concentration in free flowing bile, becoming the predominant bile acid only after one or more days of enterohepatic cycling. However, the actual role of enteric passage or microorganisms remains uncertain, since relative biliary levels of labeled 3 α , 7 α , 12 α -trihydroxycholanolic acid were similar in both free flow and continuous enterohepatic cycling experiments during the first 9 hours (Fig. 2, 3). Such data suggest that intra-enteric conversion of

"neutral sterols" and the other 2 bile acids to 3 α , 7 α , 12 α -trihydroxycholanolic acid, if any, was not marked.

The long persistence of a relatively high concentration of labeled 3 α , 7 α , 12 α -trihydroxycholanolic acid following injection of cholesterol-4-C¹⁴ indicated that it is the predominant end-product of cholesterol metabolism in the bile of adult guinea pigs. According to Bergström(3) 12-hydroxylation of bile acid precursors in the rat do not occur after the side chain has been oxidized. Comparable to the effects of hyperthyroidism in rat (5) the heightened metabolic processes in rapidly growing young guinea pigs may favor the rapid side chain oxidation resulting in elaboration and excretion of dihydroxycholanolic rather than trihydroxycholanolic acid.

The rapid enteric passage and biliary excretion of conjugated bile acids was not unexpected with large circulating enterohepatic pool of bile acids and rate of bile acid conversion from cholesterol in the guinea pig was similar to that reported in other animals(3). The augmented cholesterol to bile acid conversion observed in animals with non-replaced loss of bile acids from fistulae(3) was minimized in the present study by substitution of normal bile for that removed from the fistulae.

After the marked depletion of 3 α , 7 α , 12 α -trihydroxycholanolic and other bile acids from the enterohepatic pool by flow from a biliary fistula, bile acids would be replaced by 3 α , 7 α -dihydroxycholanolic and 3 α -hydroxy, 7-ketocholanolic acid and only small amounts of 3 α , 7 α , 12 α -trihydroxycholanolic acid. If the failure of replacement or cycling led to continued loss of bile acids from fistula, values

for 3 α , 7 α , 12 α -trihydroxycholanolic acid in the bile would be minimal.

Danielsson and Kazuno(6), have suggested that 3 α -hydroxy, 7-ketocholanolic acid was derived from 3 α , 7 α -dihydroxycholanolic acid in the guinea pig intestine. The present study indicates that both of these bile acids as well as 3 α , 7 α , 12 α -trihydroxycholanolic acid can be derived from tissue conversion of cholesterol.

Summary. In adult guinea pigs with biliary tract fistulae and stable enterohepatic bile acid pool, cholesterol-4-C¹⁴ was converted into 3 labeled bile acids (3 α , 7 α , 12 α -trihydroxycholanolic, 3 α , 7 α -dihydroxycholanolic and 3 α -hydroxy, 7-ketocholanolic acid). This conversion occurred independently of enteric passage, 3 α , 7 α , 12 α -trihydroxycholanolic (cholic) acid appearing in relatively low but gradually increasing concentration during the first 9 hours of free flow and of continuous cycling. After one or more days of continuous enterohepatic cycling, 3 α , 7 α , 12 α -trihydroxycholanolic acid was the predominant labeled bile acid, maintaining a relatively high level until termination of the experiment 18 days after injection of cholesterol-4-C¹⁴.

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Inhibition of Vitamin B₁₂ Absorption by Bile.* (26278)

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How intrinsic factor reacts to promote efficient absorption of B₁₂ from the gastrointestinal tract is just now being fully elucidated(1). Since it has taken milligram amounts of purified intrinsic factor to catalyze the absorption of microgram amounts of B₁₂, it is possible that intrinsic factor may be destroyed by substances in the gastrointestinal tract or it may react with substances other than B₁₂(2). These substances would be inhibitors of B₁₂ absorption and are likely produced by the intestinal mucosa since ingestion of B₁₂ in the fasting state does little to improve absorption. Measuring liver concentration of absorbed B₁₂ in rats, we have found that bile is a potent inhibitor of B₁₂ absorption and this inhibition is corrected by whole gastric juice.

Methods. Rats weighing 160 g were given a 5% glucose in normal saline diet for 48 hours prior to operation. During ether anesthesia a silk ligature was placed around the duodenum one cm above or below the entrance of the bile duct. Following this, various solutions containing 0.03 μ g Co⁶⁰ labeled B₁₂ were instilled into the duodenal lumen just distal to the ligature. The rats were deprived of food and water for 6 hours and were sacrificed at 6 hours. The liver was removed and washed in soapy water after which excess blood and water was expressed by blotting. The livers were then divided into 2 nearly equal parts, placed in test tubes which were then counted in a scintillation well detector. Radioactivity of each portion was summed to give total liver radioactivity. Amount of radioactivity in the liver was determined to a 5% average error. One of the following 4 solutions was instilled into the intestine: Control solution containing 0.03 μ g of B₁₂ alone; 0.5 ml of whole rat gastric juice and B₁₂; 0.5 ml rat bile and B₁₂; 0.5 ml of

whole rat gastric juice, 0.5 ml of rat bile and B₁₂. All solutions were made up to a volume of 1 ml prior to instillation.

The rat gastric juice was obtained after the rats had been given a glucose and saline diet for 48 hours. The pylorus was ligated and 2 hours later the rat was sacrificed and the gastric juice harvested. The bile was obtained by cannulating the bile duct of a rat and allowing 12 hours for collection. The rat took in 5% glucose in normal saline *ad lib.* during bile collection. The gastric juice and bile were used within 24 hours of their collection. The pH of the bile was around 9 while the gastric juice had a pH of about 1. In the volumes used duodenal mucosa neutralized these solutions upon contact.

Results. Table I shows mean and standard error of mean liver concentration of Co⁶⁰ B₁₂ obtained from these animals under the various conditions of this experiment. In the control group a 50% decrease in liver Co⁶⁰ B₁₂ occurred in the animals when the ligature was placed above the entrance of the bile duct. This decrease in B₁₂ absorption could be prevented by addition of 0.5 ml of whole gastric juice. Instillation of 0.5 ml bile reduced liver uptake of both sets of animals. Mixing whole gastric juice with the bile prior to instillation returned the absorption to control levels.

Discussion. These data show that, in the rat, bile is an inhibitor of B₁₂ absorption since injection of bile or allowing it to enter the duodenum during absorption of B₁₂ markedly decreases the amount of B₁₂ absorbed by the intestine and concentrated by the liver. Addition of 0.5 ml of whole gastric juice is capable of correcting the absorptive defect produced by a similar volume of rat bile. In the absence of bile, gastric juice in the volume tested failed to improve B₁₂ absorption. Rat bile and bile of other species contains B₁₂(3,4). The rat bile used in these studies was assayed for B₁₂ content by

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TABLE I. Effect of Bile and Gastric Juice on Absorption and Liver Concentration of $\text{Co}^{60}\text{B}_{12}$ by the Intact Rat.

	No. of animals	Relationship of ligature to bile duct		No. of animals
		Above	Below	
Control	30	4.05 $\pm$.33	7.50 $\pm$.27	27
.5 ml gastric juice	20	6.65 $\pm$.27	7.50 $\pm$.36	20
.5 " bile	25	1.76 $\pm$.42	1.74 $\pm$.21	28
.5 " " + .5 ml gastric juice	20	4.98 $\pm$.30	6.90 $\pm$.60	25

Each figure is mean $\pm$ stand. error of mean in $\mu\text{g} \times 10^{-4}$ of B_{12} .

Dr. Paul S. Hagens using *Euglena gracilis*. His data showed that this lyophilized bile contained 95 μg of B_{12} per g of solids. This compares to a value of 4 $\mu\text{g}/\text{g}$ in dog bile (5). Therefore, one ml of bile, 27 mg of solids, contains 8% as much B_{12} as was used in these experiments.

These experiments throw no light on why bile causes inhibition of B_{12} absorption in these animals; however, non-specific inhibitory binding of B_{12} would explain the results. It is known that bile will bind B_{12} (6). In these experiments, amount of intrinsic factor necessary to improve B_{12} absorption seems related to amount of bile present in the upper intestine. This effect would in part explain why isolation of potent pure intrinsic factor has been so difficult since minimal effective dose of intrinsic factor may be more related to the bile in the intestine than to amount of vitamin B_{12} ingested. It is possible that

other intestinal secretions inhibit B_{12} absorption.

Summary. 1. In the intact rat the presence of bile in the small intestine inhibits B_{12} absorption. 2. The inhibitory effect of bile is prevented by simultaneous addition of rat gastric juice.

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Gamma Globulin and Immune Mechanisms in the Chick Embryo.* (26279)

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Green and Lorincz(1) stated that a serum protein with the properties of a natural antibody first appeared in the blood of chick embryos around the 17th day of embryonic development. This antibody, which is a part of the serum gamma globulin, seems to be responsible for destruction of the Krebs as-

cites tumor cells of mice injected intravenously into the chorio-allantois of chick embryos. Mouse tumor cells do not grow in hatched chicks. In connection with our findings(2) on the fate and behavior of mammalian skin heterografts transplanted to the chorio-allantois of the chick embryo, it was a natural sequence to learn whether gamma globulin placed directly on the embryonal membrane, or injected intravenously into the

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chorionic blood vessels, would have any possible influence on skin heterografts transplanted to the chorio-allantoic membrane. This investigation could also furnish additional information on the role of the serum gamma globulin in immune mechanisms.

Methods and technics. Skin grafts were taken from the flanks of rabbits and guinea pigs, and divided into pieces approximately 10×10 mm in size. The technic of preparation of the 10-day-old fertile chick eggs and application of the skin grafts to the membrane has been described(3).

Whole blood containing anti-rabbit gamma globulin was pooled from a group of hens previously immunized with rabbit spleen suspension. Serum protein fractions were obtained by the technic of Green and Lorincz(1) and final protein concentration of anti-rabbit gamma globulin was determined as 109 mg/ml. Anti-guinea pig gamma globulin was prepared in the same fashion. Concentration was 50.5 mg/ml. The guinea pig antibody fractions were found to agglutinate normal guinea pig red blood cells at 1:1,000 dilution.

The technic of Beveridge and Burnet(4) was used for injecting gamma globulin intravenously into the chorionic vessels of 85 chick embryos immediately prior to skin heterografting in a first series. In a second series of 104 embryos the gamma globulin was placed on the chorio-allantoic membrane adjacent to the skin heterografts. In a third control series 99 embryos received viable skin heterografts and no gamma globulin.

The mortality after grafting 288 eggs was 30%, but bacterial and mycotic infections caused an additional 15% loss despite the use of appropriate antibiotics. The eggs were sacrificed on the 4th, 5th, and 6th post operative days for histological sectioning. Daily stereomicroscopic examinations were made to determine viability of the grafts.

Results. The control eggs with skin heterografts and no gamma globulin exhibited the histologic and stereomicroscopic findings previously reported by us(2). Penetration of the chick blood vessels into the dense dermis of the guinea pig skin heterograft was not as deep or as extensive as in the rabbit skin heterograft.

After intravenous injection of 0.04 to 0.06 ml (2.0-3.0 mg) of concentrated guinea pig antibody serum no obvious effect on fate or behavior of the implanted guinea pig skin grafts could be observed either by stereomicroscopic or histologic observation. When dosage was increased to 0.15 ml (7.5 mg) no visible changes of the guinea pig grafts were observed.

The topical application of 0.05 ml of anti-rabbit gamma globulin (5.0 mg) to the ectodermal surface of the chick membrane failed to elicit any accelerated rejection of the rabbit skin grafts. There was no response to the rabbit skin graft after direct placement of 0.06 ml (3.0 mg) of guinea pig protein fractions to the chorio-allantois of the chick embryo.

Use of rabbit and guinea pig anti-serum by either direct application or intravenous injection appeared to evoke no significant cellular activity within the membranal stroma underlying the graft. Compared with the controls, there was no significant increase in infiltration of leucocytic, lymphoid-type, or large round cells with vesicular nuclei in the mesoderm as described by Billingham and Silvers (5).

Discussion. Administration of immune gamma globulin into the chick chorio-allantoic vessels or to the ectodermal surface of the membrane fails to produce detectable damage to skin heterografts.

Green and Lorincz(1) demonstrated that when a large number of tumor cells were injected intravenously into the chorionic vessels of the chick embryo none of the chicks succeeded in removing the cells. This suggests that the quantity of skin heterograft is too great to be effectively influenced by immune fractions. The areas where the foreign tissues are placed, whether within the embryo, or on the chorio-allantois, or in the yolk sac, may be responsible for the differences in immunological responses. Perhaps when the graft is situated on the chorio-allantoic membrane, the blood supply is not sufficiently great to furnish adequate antibody or complement for the cytolytic effect. Green and Lorincz(1) also postulated that the larger the quantity, the more resistant the tissue

was to a fixed quantity of antibody and that the degeneration depended on whether the tissue was growing within the body of the embryo or in the yolk sac. The nature of the tissues used (*i.e.*, skin, organs, cornea, etc.) and the species from which the tissues are obtained are also 2 important considerations. Billingham, Brent, and Medawar(6) demonstrated that the power to react against different antigens may mature at different times. According to these authors, "Birth itself is no guide to the time of origin of immunologic maturity."

Summary. The histologic findings reveal that rabbit and guinea pig anti-serum placed directly on the chick embryonal membrane or injected intravenously into the chorionic vessels fails to produce any detectable changes to skin heterografts transplanted to

the chorio-allantoic membrane.

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Utilization of Injected and Orally Administered Amino Acids by the Rat.* (26280)

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In an effort to determine whether the gastrointestinal tract was an important site of the leucine-isoleucine antagonism in the rat (1,2,3), the effectiveness of parenterally administered isoleucine and valine in preventing the growth retardation due to a dietary excess of L-leucine was compared with that of isoleucine and valine included in the diet. Initial results indicated that the parenterally administered amino acids were ineffective, so the ability of parenterally administered isoleucine to prevent a simple isoleucine deficiency was determined. The growth response of rats fed an isoleucine-deficient diet and injected with isoleucine differed from the response of rats fed a lysine-deficient diet

and injected with lysine. These observations and others on the effects of dietary and parenterally administered isoleucine and valine in overcoming growth retardation due to an excess of dietary L-leucine are reported.

Experimental. Male rats of the Holtzman strain were used throughout these experiments. Except for experiments on rats trained to eat a single meal daily, each group consisted of 6 rats weighing between 100 and 110 g and was fed *ad libitum*. Rats trained to accept a single daily feeding were fed a 15% casein-dextrin diet for only 2 hours each day for a period of 2 weeks(4). Their body weights at start of the experiments were between 210 and 230 g. Average initial weights for the groups within each experiment did not differ by more than one gram. All rats were supplied with water *ad libitum*, were housed in individual cages with raised screen bottoms and were weighed at a regular time daily.

All of the diets contained salt mixture, 5%

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(5); corn oil, 5%; and vitamins in mg/100 g of diet as follows: thiamine, 0.5; riboflavin, 0.5; niacin, 2.5; calcium pantothenate, 2.0; pyridoxine, 0.25; biotin, 0.01; folic acid, 0.02; Vit. B₁₂, 0.002; inositol, 10.0; Vit. C, 5.0; choline chloride, 150; Vit. A, 400 IU; Vit. D, 200 IU; and α -tocopherol, 10.0. The fat soluble vitamins were dissolved in the corn oil.

The lysine-deficient diet contained 30% of wheat gluten and dextrin as the carbohydrate to make up 100%; 0.4% of L-lysine · HCl was included in the control diet(6). The isoleucine-deficient diet contained 18% of blood meal and 0.25% of DL-isoleucine with dextrin to make up 100%; 1% of DL-isoleucine was included in the control diet(1). The amino acid diet contained all of the amino acids in the quantities that would be provided by 9% of casein except that values for L-isoleucine and L-valine were reduced by one-third. Values from Block and Weiss(7) were used for the amino acid composition of casein. Sucrose was used as the carbohydrate in this diet and was included to make up 100%; the supplemented amino acid diet contained adequate amounts of isoleucine and valine. The high leucine diet contained casein, 9%; DL-methionine, 0.3%; L-glutamic acid, 0.74%; and L-leucine, 5%; the supplemented diet contained L-isoleucine, 0.325%; and L-valine, 0.30%; instead of the glutamic acid(3).

All amino acid solutions used for injections were isotonic (0.306 osmolar) and the pH was adjusted to 7.5.

Food consumption of individual rats was recorded daily and volume of the amino acid solution to be injected was calculated to provide each rat with a slightly larger quantity of amino acids each day than was consumed by rats receiving the supplemented diet.

In the *ad libitum* feeding experiments animals were injected twice daily at 8:30 a.m. and at 5:30 p.m. Subcutaneous injections were made under the skin on the back of the necks of the animals and intravenous injections into the tail vein. Control animals were injected with an equal volume of physiologic saline solution. Trained rats were in-

jected once daily at the end of the 2-hour feeding period.

Results. A comparison of effects of administering lysine in different ways to rats fed *ad libitum* a diet deficient in this amino acid is shown in Fig. 1. Rats receiving 0.4% of lysine in the diet grew an average of 50 g more in 10 days than rats fed the unsupplemented diet. When lysine was administered by injection twice daily to rats receiving the unsupplemented diet, rate of growth was approximately half that of rats receiving lysine in the diet. Growth rates of rats injected subcutaneously or intraperitoneally were the same; both grew an average of 24-26 g more than the group fed the unsupplemented diet. The difference between the growth of the group injected with lysine solution and that of the group receiving the unsupplemented diet and injected with saline solution was highly significant ($P < .001$).

A comparison of the effects of administering L-isoleucine and L-valine to rats fed the diet containing an amino acid mixture that was low in these 2 amino acids is shown in Fig. 2. These animals like those in the previous experiment were fed *ad libitum*, and were injected subcutaneously twice daily. Rats receiving the complete amino acid diet grew 28 g more in 12 days than those receiving the amino acid mixture that was low in isoleucine and valine. There was no noticeable effect of the subcutaneous injections of isoleucine and valine up to 6 days. From the 6th to the 12th day the group receiving the amino acid injections grew a little more rapidly but the difference at the 12th day was not statistically significant ($P > 0.05$).

In a similar experiment with rats fed *ad libitum* an isoleucine-deficient diet containing 18% of blood meal and 0.25% of DL-isoleucine no significant difference was observed between the growth of rats injected twice daily with saline solution and that of a group injected with an isoleucine solution for a period of 19 days.

Fig. 3 summarizes results of an experiment in which rats trained to accept a single daily feeding (2 hr) were fed the isoleucine-deficient diet containing blood meal and were injected with isoleucine or saline solutions at

UTILIZATION OF INJECTED AMINO ACIDS

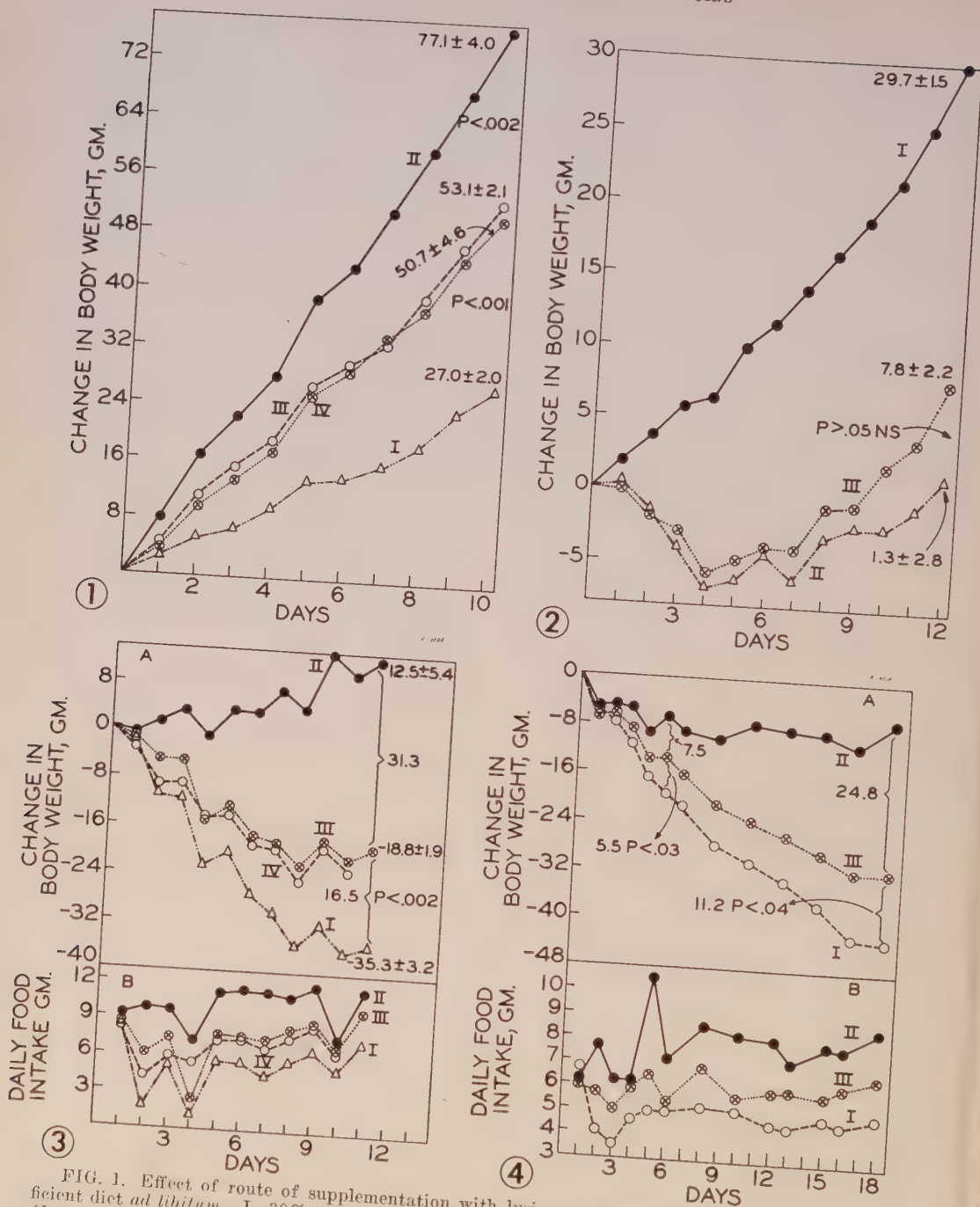


FIG. 1. Effect of route of supplementation with lysine on growth of rats fed a lysine-deficient diet *ad libitum*. I. 30% wheat gluten-dextrin diet, inj. with saline solution; II. as for (I) + 0.4% L-lysine • HCl in the diet, inj. with saline solution; III. as for (I) + 0.4% L-lysine • HCl inj. intraper.; IV. as for (I) + 0.4% L-lysine • HCl inj. subcut.

FIG. 2. Effect of route of supplementation with isoleucine and valine on growth of rats fed *ad libitum* a diet deficient in these 2 amino acids. I. Amino acid diet containing amino acids in quantities in 9% of casein; inj. with saline solution; II. as for (I) except that quantities of L-isoleucine and L-valine were reduced by one-third, inj. with saline solution; III. as for (II) + L-isoleucine and L-valine inj. subcut.

the end of the feeding period. The groups receiving isoleucine supplement by subcutaneous or intravenous injection grew significantly faster ($P < .002$) than those given injections of saline solution. However, the response of rats receiving isoleucine injections was only one-third that of rats receiving isoleucine in the diet.

In Fig. 4 are summarized the results of an experiment in which rats trained to eat a single meal daily were fed a diet high in leucine (9% of casein + 5% L-leucine). Isoleucine and valine were included in the diet of one group, and another received these amino acids by subcutaneous injection at the end of feeding period as in the previous experiment. Isoleucine and valine injections produced a significant growth response ($P < .04$) but again it was only one-third as great as that obtained when the amino acids were included in the diet. The response was significant ($P < 0.03$) by the 5th day.

A further point of interest is that rats trained to eat a single meal daily (2 hours feeding every day) ate the same quantity of the isoleucine-deficient or the isoleucine-supplemented diet on the first day, but on the second day and thereafter the group receiving the isoleucine-deficient diet ate much less (Fig. 3). Rats fed the high-leucine diet or the high-leucine diet supplemented with isoleucine and valine (Fig. 4) also ate equal amounts on the first day and only on the second day did the food intake of the group receiving no extra isoleucine and valine fall. The appetite-depressing effects of isoleucine-deficiency and of excess leucine appear, therefore, to result from metabolic disturbances and not from low palatability of the diets.

Discussion. The observation that parenterally administered lysine is used more efficiently by the rat than parenterally admin-

istered isoleucine emphasizes that rates of catabolism of the individual amino acids in animal tissues differ considerably and that such differences are of much more significance when amino acids are administered parenterally.

Utilization of parenterally administered tryptophan(8), threonine(9) and lysine(10) has been reported but no systematic study of the relative rates of catabolism of individual amino acids administered by both routes appears to have been made.

Dalgliesh and Tabechan(11) observed that the overall rates of degradation of phenylalanine and tryptophan were similar, and about one-half that of tyrosine in the rat. Using chicks, Ousterhout(12) found the greatest rate of oxidation for labeled valine, this was followed by histidine, and threonine had the slowest rate of oxidation. Wu *et al.* (13,14) reported that concentration of N^{15} in ammonia and urea was higher after administration of labeled aspartate to human subjects than after administration of phenylalanine. Borsook *et al.*(15) found that mice injected with labeled amino acids oxidized 57% of the injected activity from leucine, 44% of that from histidine, 28% of that from glycine and 27% of that from lysine to expired CO_2 within 4 hours. Radioactivity in the non-protein fraction of the blood disappeared within one hour after glycine, leucine and histidine administration but a substantial amount of radioisotope was found in the 4-hour sample of blood from mice receiving lysine- C^{14} . Doty and Eaton(16) injected fasted dogs with glycine or lysine and observed that blood urea-nitrogen increased much less after lysine than after glycine administration. Bender(17) using rats obtained a much higher value for the net protein utilization of a lysine-free diet than for

FIG. 3. Effect of route of supplementation with isoleucine on growth of rats fed an isoleucine-deficient diet for only 2 hr daily. A: Growth; B: Food consumption. I. 18% blood meal + 0.25% DL-isoleucine in diet, inj. with saline solution; II. 18% blood meal + 1.0% DL-isoleucine in diet, inj. with saline solution; III. as for (I) + 0.75% DL-isoleucine inj. subcut. at end of feeding period; IV. as for (I) + 0.75% DL-isoleucine inj. intrav. at end of feeding period.

FIG. 4. Effectiveness of subcut. injections of L-isoleucine and L-valine in overcoming growth-retarding action of excess leucine in rats fed for only 2 hr daily. A: Growth; B: Food consumption. I. 9% casein + 5% L-leucine in diet, inj. with saline solution; II. as for (I) + 0.325% L-isoleucine + 0.30% L-valine in diet, inj. with saline solution; III. as for (I) + 0.325% of L-isoleucine + 0.30% of L-valine inj. subcut. at end of feeding period.

diets lacking any of the other indispensable amino acids. He also reported that animals survived for more than 6 months on a lysine-free diet.

Studies of the effects of delayed oral supplementation of diets with amino acids have led to the concept that for efficient synthesis of tissue proteins all of the essential amino acids must be supplied to the organism at close to the same time(10,18-23). The necessity for simultaneous availability of all of the essential amino acids indicates that amino acids are not stored to any appreciable extent in the body. However, in several experiments in which plasma amino acid concentrations of fasted dogs(24), fasted rats(25) and fasted chicks(26) were determined values for threonine and lysine were consistently higher than those for other indispensable amino acids. This may be further evidence of a slow rate of catabolism of these 2 amino acids and help to explain the relatively efficient utilization of injected lysine.

The present results indicate that differences in the extent of catabolism of individual amino acids may be greatly magnified if the amino acids are administered parenterally rather than fed as components of the diet. An awareness of this factor is important in studies of amino acid metabolism.

Summary. Rats fed a lysine-deficient diet *ad libitum* and injected subcutaneously twice daily with a lysine solution grew at about half the rate of rats receiving lysine in the diet. Rats fed *ad libitum* an isoleucine-deficient diet failed to respond when they were injected twice daily with an isoleucine solution. If rats were fed the isoleucine-deficient diet for a period of only 2 hours daily and were given subcutaneous injections of isoleucine at the end of feeding period their rate of growth was about one-third that of rats receiving isoleucine in the diet. Under the same conditions, injection of rats fed a diet high in leucine with isoleucine and valine resulted in a comparable improvement of growth. These observations indicate that the rate of catabolism of isoleucine is much faster than that of lysine in the rat and that the amount of an amino acid catabolized de-

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Interactions of Triethylenethiophosphoramide, Estrogen and Gonadotropin On the Ovary of Hypophysectomized Immature Rat.* (26281)

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The dynamic morphologic changes occurring in the ovary during the normal ovarian cycle and under experimental conditions have been reviewed by Hisaw(1). Interference with these processes by cytotoxic agents which act most effectively on rapidly proliferating cells offers interesting avenues of study. The present report is concerned with disturbances provoked by triethylenethiophosphoramide (Thio-TEPA) on the ovary of the hypophysectomized immature rat and the influences of a pituitary gonadotropic preparation and diethylstilbestrol on such effects.

Methods. The experimental animals were immature rats of the Holtzman strain, hypophysectomized at age of 24 days. Injections were begun immediately after hypophysectomy and repeated daily for a total of 4 doses. At autopsy, 48 hours after final injection, the ovaries were quickly weighed, preserved in 10% buffered formalin and subsequently prepared for microscopic study by the standard Hematoxylin and Eosin technic. Ovarian weight was corrected to a final body weight of 100 g.

Thio-TEPA in a saline solution was administered intraperitoneally. The partially purified sheep anterior pituitary gonadotropin (Gonadophysin®) was administered subcutaneously in doses of .2 mg dissolved in .2 ml of saline. Diethylstilbestrol in doses of 1 mg dissolved in .1 ml of sesame oil was injected subcutaneously.

Results. Thio-TEPA administered alone to the hypophysectomized animals produced

a slight but quantitative reduction in ovarian weight. The intense stimulation of increased ovarian weight by relatively large doses of estrogen or pituitary gonadotropin was more precipitously antagonized by Thio-TEPA. Maximal ovarian stimulation with the combination of gonadotropin and estrogen provided the most striking example of quantitative inhibition of ovarian growth produced by increasing doses of the antineoplastic agent (Table I).

Thio-TEPA alone in the smaller doses produced destruction of granulosa cells and follicular atresia, predominantly in the larger follicles. However, as the dose was increased this effect became more evident in smaller follicles. Destruction of primordial follicles

TABLE I. Effects of Thio-TEPA on Ovarian Weight in Hypophysectomized Immature Rat.

Thio-TEPA	Treatment*		No. of		Mean ovarian wt, mg/100 g body wt ± S.E.
	DES	Gonadophysin	Animals	Survivors	
.1			10	10	18.07 ± .45
.15			3	3	15.65
			16	8	14.20 ± .97
	1		14	14	63.95 ± 2.20
.05	1		3	3	56.20
.1	1		18	11	44.20 ± 1.33
.15	1		23	12	39.20 ± 3.42
.2	1		4	1	25.70
		.2	5	5	41.68 ± 5.92
.1		.2	11	11	38.60 ± 3.63
.15		.2	14	11	31.80 ± 4.16
	1	.2	18	18	167.20 ± 14.87
.1	1	.2	15	13	91.21 ± 4.70
.15	1	.2	19	13	68.20 ± 5.35
.2	1	.2	4	1	40.60

* Daily dose in mg administered for a total of 4 doses beginning immediately after hypophysectomy.

* Supported by U.S.P.H.S. grant.

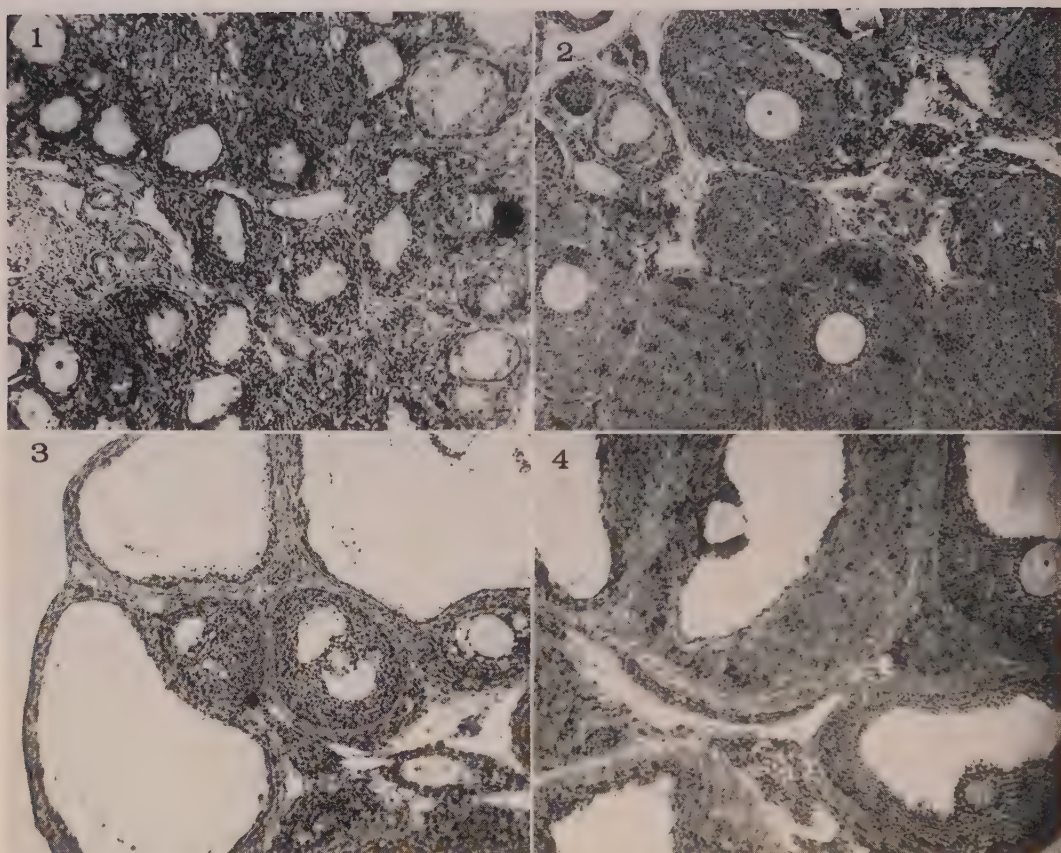


FIG. 1-4. Ovaries of hypophysectomized immature rats treated with 4 daily doses of indicated materials beginning immediately after hypophysectomy, with autopsy 48 hr after last inj. All magnifications $\times 60$.

FIG. 1. .15 mg Thio-TEPA. Ovary is small. Follicular atresia and necrosis of ova in larger follicles. Primordial follicles unaffected.

FIG. 2. .15 mg Thio-TEPA + 1 mg DES. Follicular development to medium sized solid stage. Slight loosening of granulosa cells and early follicular atresia with an occasional necrotic ovum. Only sparse thecal and interstitial tissue.

FIG. 3. .15 mg Thio-TEPA + .2 mg gonadotropin. Widespread dissolution and pyknosis of granulosa cells, with atresia of follicles and cystic follicle formation. Most ova necrotic. Slight stimulation of thecal and interstitial tissue.

FIG. 4. .15 mg Thio-TEPA + .2 mg gonadotropin + 1 mg DES. Partial protection of granulosa cells and ova. Luteinization has occurred. Thecal and interstitial tissue are stimulated.

occurred only with the largest dose of Thio-TEPA. Necrosis of the ovum in the atretic follicle was generally apparent (Fig. 1).

Diethylstilbestrol (DES), in the large dose used, provided maximal development of ovarian follicles in the hypophysectomized immature rat as previously described(2). Such treatment with DES afforded substantial protection against the cytotoxic effects of Thio-TEPA on granulosa cells and ova (Fig. 2). An increased number of primordial follicles was present in the Thio-TEPA-DES ovaries

in contrast to those treated with DES alone.

When gonadotropin in the dose employed in this study was administered alone to the hypophysectomized rat prominent vesicular follicles developed with occasional luteinization, as previously described(4). When Thio-TEPA in increasing doses was administered concurrently progressive atresia of developing follicles was observed, with destruction of granulosa cells and ova. Under these circumstances cystic follicles were formed (Fig. 3). Such ovaries resemble those produced

by small doses of pregnant mare serum gonadotropin in the hypophysectomized rat(3). Stimulation of thecal and interstitial cells by gonadotropin was only questionably impaired by Thio-TEPA and the observed narrowing of these zones was largely accountable to compression by encroachment of the cystic follicles. A rim of granulosa cells frequently persisted lining the membrana granulosa but no evidence of luteinization was seen in these cells.

When DES was added to gonadotropin-Thio-TEPA treatment, granulosa cells and ova were protected, and luteinization proceeded. Thio-TEPA appeared to increase the tendency for 'premature' luteinization, *i.e.*, luteinization of the follicle prior to ovulation, and to hasten atresia of the small solid follicles (Fig. 4).

Discussion. Cytotoxic effects of Thio-TEPA appeared to be directed primarily against rapidly proliferating granulosa cells. Destruction of ova and inhibition of cell growth in the theca interna and interstitial tissue were considered secondary to effects produced on granulosa cells. Thickening of a 'restricting' membrana granulosa under the influence of Thio-TEPA would also appear to be a secondary effect of the agent. Burkl and Kellner(5) have described thickening of the membrana granulosa as the preponderant disruptive effect of busulfan (Myleran®) on the ovary of the intact immature rat. Formation of cystic follicles without luteinization in the Thio-TEPA-gonadotropin treated animals may similarly represent an induced defect in membrana granulosa dissolution, offering speculation as to mechanisms by which cystic follicles are formed. However, such ovaries do not show development of theca interna and interstitial tissue to the degree that occurs following administration of gonadotropin alone, which might be attributed to crowding by the cystic follicles.

Protection of granulosa cells against Thio-TEPA by DES is of particular interest in view of intense stimulation of these cells by the estrogen. One would rather expect that such rapidly proliferating cells would be particularly susceptible to Thio-TEPA as has been reported with cells in tissue culture(6).

It would be of interest to determine whether a similar protective effect of estrogen against Thio-TEPA applies to other cells.

Estrogen does not stimulate ovarian thecal or interstitial cell proliferation in the hypophysectomized rat(2). The present study demonstrates that DES facilitates luteinization in gonadotropin-Thio-TEPA treated hypophysectomized animals. The mechanism responsible for this effect is assumed to be a protective effect of DES on the follicle until follicular events are conducive to luteinization. It is of further interest that DES, in presence of gonadotropin stimulation and Thio-TEPA destruction, appears to promote direct luteinization of granulosa cells as manifested by premature luteinization.

Intense estrogen stimulation in the hypophysectomized rat slows formation of primordial follicles while producing an abundance of medium-sized solid follicles(2). The presence of primordial follicles in the Thio-TEPA-DES ovaries would indicate either persistence or increased production of primordial follicles by antagonism to development of larger follicles.

Summary. In the hypophysectomized weanling rat triethylenethiophosphoramidate (Thio-TEPA) produced quantitative destruction of granulosa cells and ova. Stimulation of the ovary by pituitary gonadotropin was antagonized by Thio-TEPA, resulting in blocking of luteinization and formation of cystic follicles. Diethylstilbestrol partially protected against destructive effects of Thio-TEPA on the ovary allowing luteinization to proceed. Under all circumstances Thio-TEPA caused a quantitative reduction in ovarian weight over a period of 5 days.

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Inhibitory Effect of Human Blood Components on a Lipase of Microbiological Origin.* (26282)

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Investigation of lipid metabolism in patients with hyperlipemia raised the possibility of presence of lipase inhibitors in plasma. Tauber(1) reported the presence of antilipase activity in human, rabbit and guinea pig serum and in laked rabbit red blood cells against the lipolytic activity found in rabbit serum. In the present study, it was found that normal human plasma and red blood cells inhibited lipolytic activity of a lipase of microbiological origin. An attempt was made to determine in what fractions of blood this inhibitory activity resided.

Materials and methods. Olive oil was used as substrate and a mixture of 3 ml of a stable olive oil emulsion and 0.5 ml of a phosphate buffer at pH 6.56, as described by Tauber (1), was used for each experiment. The results in Table I were obtained using one mg of a microbiological lipase (Lipase B, Mix 4A, Rohm and Haas Co.)[†] and incubating at 37°C with shaking for 20 hr, and in Tables II and III using 10 mg of a microbiological lipase (Mann Research Laboratories, Inc.)[†] and incubating at 37°C with shaking for 1.5 hr. Following incubation, 2 drops of phenolphthalein and 3 ml of 95% ethanol were added, and the mixture was titrated with NaOH.

Freshly drawn blood from normal non-fasting adults was used. The red blood cell hemolysate was made by adding 5 ml red blood cells to 6 ml water and the red blood cell ghosts by hemolyzing 5 ml red blood cells in water, washing the centrifugate 4 times with water, and resuspending in 10 ml water. The hemoglobin solution contained 0.15 g per ml water. The red blood cell dialysate was made by adding 12 ml red

blood cells to 12 ml water in a cellophane dialysis bag which was allowed to stand in 30 ml water at 4°C for 19 hr. After removing dialysate, the bag was dialyzed in running water for 30 min. The plasma dialysate was made similarly, using 3 ml plasma against 40 ml water. The ashed solutions were remade to original volume.

Results. Both plasma and a red blood cell

TABLE I. Effect of Human Blood Components on Lipase Activity.

Sample	Vol, ml	Before* After*	
		—ml NaOH—	
RBC hemolysate	.5	.94	4.30
" "	.1	.96	—
" ghosts	.5	7.58	7.69
Hemoglobin	.5	1.35	3.64
RBC-d.	2.0	4.30	6.80
" heated†	2.0	5.08	6.71
" ashed	2.0	5.33	6.68
RBC-n.	2.0	1.16	5.84
" heated†	2.0	1.94	4.72
" ashed	2.0	6.75	6.58
WBC-1§	.5	6.26	6.85
" 2§	.5	5.58	5.57
Plasma	1.0	1.44	7.93
" no enzyme	1.0	1.25	—
Plasma-d.	2.0	5.96	7.76
Plasma-n.	.5	2.00	8.27
" heated†	1.0	4.85	8.16
Serum-heated†-supernatant	.5	5.48	7.17
" precipitate	.5	1.99	7.19
Water	2.0	5.16	6.80
" no enzyme	2.0	1.00	—
Enzyme control†	—	7.54	1.47
No enzyme control	—	1.15	—

* Titration values in ml 0.046 N NaOH for addition of sample before and after incubation.

† Titration values are for addition of enzyme before and after incubation of the emulsion and buffer mixture.

‡ Heated at 100°C for 8 min.

§ WBC-1 prepared by Method #2 of Walford *et al.*(2). (WBC from 40 ml blood suspended in 7 ml isotonic saline); WBC-2 prepared by method of Lapin *et al.*(3) (WBC from 35 ml blood suspended in 7 ml isotonic saline).

|| Contained emulsion and buffer only.

Abbreviations are as follows: RBC—red blood cells; d.—dialysate; n.—non-dialyzable fraction; WBC—white blood cells.

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† Prepared from a species of fungus in the *Aspergillus oryzae-niger* group.

TABLE II. Effect of Human Plasma Protein Fractions on Lipase Activity.

Fraction	Before*	After*
	—ml NaOH—	
I	3.88	7.97
II	4.60	7.25
III-0	4.50	7.35
III	4.22	7.57
IV-1	4.40	7.78
IV-4	3.78	7.35
V	4.07	7.79
Enzyme control†	8.10	2.08
No enzyme control‡	1.80	—

Fifty mg of each fraction was added in 0.5 ml water. Fractions and descriptions thereof were generously supplied by Dr. R. B. Pennell, Protein Foundation, Inc., Jamaica Plain, Mass. Composition of these fractions is as follows: I—fibrinogen, antihemophilic globulin, cold insoluble globulin, etc.; II—gamma globulin; III-0— β -lipoprotein; III—clotting components, isoagglutinins, plasminogen, etc.; IV-1— α -lipoprotein, ceruloplasmin; IV-4— β -metal combining protein, serum esterase, α -lipoprotein, albumin, etc.; V—albumin. Fractions I, IV-1, IV-4, and V were prepared by the method of Cohn *et al.*(4); fractions II, III-0, and III were prepared by the method of Oncley *et al.*(5), using as starting material fractions II + III obtained by the former method.

* Titration values in ml .047 N NaOH for addition of fraction before and after incubation.

† Titration values for addition of enzyme before and after incubation of the emulsion and buffer mixture.

‡ Contained emulsion and buffer only.

hemolysate inhibited enzyme activity, whereas white blood cells did not (Table I). White blood cells showed no appreciable lipolytic activity when incubated with the emulsion and buffer only, even after sonication and addition of taurocholate; thus, an inhibitory effect did not appear to be masked by any lipolytic activity of the white blood cells. Red blood cell ghosts appeared to have no effect on enzyme activity, but a hemoglobin preparation showed inhibitory activity. The non-dialyzable portion of a red blood cell hemolysate showed inhibitory activity, which was destroyed by ashing but not by heating at 100°C for 8 min; the dialysate appeared to have some inhibitory activity, which was destroyed by heating at 100°C for 8 min and by ashing. The non-dialyzable fraction of plasma showed inhibitory activity which appeared to be partly destroyed by heating at 100°C for 8 min, but the dialyzable fraction appeared to be inactive. Likewise, the pre-

cipitate obtained by heating serum at 100°C for 8 min was inhibitory, but the supernatant apparently was not. No inhibitory activity was seen with urine or with heparin, which was used as anticoagulant. From Table II, it can be seen that human plasma protein fractions obtained by cold ethanol fractionation all showed some inhibitory activity. The inhibitory effect of human serum albumin appeared to be a function of its concentration (Table III). The microbiological lipase (Mann) also hydrolyzed triacetin but not Tween 20. Albumin, however, did not inhibit triacetin hydrolysis. Pork pancreatic lipase (Nutritional Biochemicals Corp.) was found to hydrolyze the olive oil emulsion, but was not inhibited by plasma, red blood cells, or albumin. Albumin, likewise, did not inhibit triacetin and Tween 20 hydrolysis by this enzyme. Wheat germ lipase (Mann) did not hydrolyze the olive oil emulsion but did hydrolyze triacetin.

The activity of both microbiological lipases showed little change when initial pH of the incubating mixture was varied over the range of 6.4 to 7.0. Addition of the protein fractions, plasma, or red blood cells did not move the pH of the mixture out of this range before incubation. Moreover, in the samples tested (red blood cells, fraction V, enzyme

TABLE III. Effect of Human Serum Albumin on Lipase Activity.

Albumin, mg	Before*	After*
	—ml NaOH—	
1.6	7.05	7.33
3.2	5.39	—
6.3	4.60	—
12.5	4.20	7.47
25.0	4.02	—
50.0	3.50	—
100.0	3.57	7.50
Saline—1 ml	7.19	6.97
Enzyme control†	7.72	1.50
No enzyme control‡	1.15	—

Each quantity of the lyophilized human serum albumin (Cutter Laboratories) used was added in 1 ml isotonic saline. Dialyzing the albumin solution did not appreciably alter the results.

* Titration values in ml 0.047 N NaOH for addition of fraction before and after incubation.

† Titration values for addition of enzyme before and after incubation of emulsion and buffer mixture.

‡ Contained emulsion and buffer only.

control), the pH did not change more than 0.1 unit as a result of incubation. This pH stability may have resulted from the insolubility of the fatty acids produced allowing little or no effect on pH. The inhibition of enzyme activity by the materials tested, therefore, could not be accounted for by an effect on pH of the incubating mixture.

Discussion. It appeared, therefore, that protein components most likely accounted for the lipase inhibition seen with plasma and red blood cells. Tauber(1) in fact, reported presence of serum antilipase which inhibited lipolytic activity normally found in some sera; he also found that laked rabbit red blood cells inhibited serum lipase activity and concluded that hemoglobin was the inhibitor. The ineffectiveness of ashed plasma and red blood cells in the present study indicated that inorganic electrolytes were not responsible for the inhibition.

The mechanism of an inhibitory activity of plasma proteins and hemoglobin on this enzyme is unknown. If the enzyme were autocatalytic, as reported for a lecithinase (6), the protein could prevent activation of the enzyme by binding the fatty acid products. The lack of albumin inhibition on triacetin hydrolysis by this enzyme, however, is evidence against this possibility, and suggests that the inhibitory effect of albumin

and the other protein fractions on enzyme hydrolysis of the olive oil emulsion might have been the result of coating of the lipid droplets by these proteins, thereby preventing access of the substrate to the enzyme.

Summary. Human plasma, red blood cells, plasma protein fractions and a hemoglobin preparation were found to inhibit lipolytic activity of a microbiological lipase preparation when an olive oil emulsion was used as substrate. Human serum albumin, which was inhibitory in proportion to its concentration, was not inhibitory when triacetin was used as substrate. Hydrolysis of the olive oil emulsion by pork pancreatic extract was not inhibited by human plasma, red blood cells or albumin.

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Influence of Age on Liver Phospholipide Metabolism of Mice.* (26283)

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Phospholipides are involved in many basic structures of the cell, constituting 16-32% dry weight of nuclei, mitochondria and microsomes(1), and are chemically part of certain enzymes(2). To our knowledge, the influence of age upon radioactive turnover and chromatographic separation of the individual

phospholipides has not been previously reported.

Methods. Alteration of phospholipide metabolism and distribution with increasing age was studied in inbred strains of female mice (AxZb). Six hours before sacrifice 25 μ c of $\text{NaH}_2\text{P}^{32}\text{O}_4$ was administered intraperitoneally. This time interval corresponds to the ascending part of the specific activity

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TABLE I. Influence of Age on Liver Phospholipide Composition and Relative Specific Activities of Mice.*

Age (days)	No. of ani- mals	Total lipid phosphorus, $\mu\text{g}/\text{mg}$ liver N	% total lipid phosphorus				
			Phos- phatidyl serine	Phosphatidyl ethanolamine	Phos- phatidyl inositol	Lecithin	Sphingo- myelin
8	8	16.93	7.94 (.20)	24.39 (.94)	20.10 (.81)	40.00 (.88)	7.92 (.46)
17	5	29.08	5.00 (.06)	30.10 (.31)	12.04 (.21)	45.44 (.42)	7.42 (.22)
24	4	26.40	3.17 (.05)	32.48 (.26)	13.74 (.15)	40.06	10.55 (.21)
60	3	36.44	2.52 (.11)	29.54 (.36)	10.00 (.21)	52.81 (.36)	5.12 (.26)
145	2	34.99	17.18 (.22)	15.41 (.26)	9.37 (.12)	51.05 (.28)	6.99 (.25)
365	3	40.41	16.06 (.19)	16.21 (.22)	8.74 (.12)	51.41 (.21)	7.59 (.16)
840	2	35.45	2.94 (.06)	30.78 (.24)	10.30 (.17)	49.14 (.20)	6.84 (.14)

* Values in parentheses are relative specific activities.

time curves, where synthesis of phospholipides occurs to a degree greater than breakdown(3). The livers were removed, pooled for each age group, and homogenized in the cold for 2 minutes in 95% ethanol in a Potter-Elvehjem homogenizer. An aliquot was taken of the homogenate for extraction of the inorganic soluble phosphorus with 10% trichloroacetic acid, and radioactivity(4) and phosphorus(5) were determined. Nitrogen was determined by the semimicro-Kjeldahl method on another aliquot. The lipides were extracted from the remainder of the homogenate, chromatographed on silicic acid columns, and eluted with chloroform-methanol by the method of Hanahan(6) using a Gilson Medical Electronics fraction collector. Radioactivity(4) and phosphorus(5) were determined on aliquots of the chloroform-methanol fractions. The composition of each of the individual phospholipide fractions was further substantiated by utilizing the paper chromatographic technic of Marinetti *et al.* (7). As a measure of phospholipide synthesis the relative specific activity has been calculated(8). This is the ratio of specific activities of phospholipide phosphorus to specific activities of acid soluble phosphorus. Under the conditions of our experiments an increase in formation of phospholipides may reasonably be assumed when higher values of relative specific activities are found. Radioactivity was determined with a ultra-thin window, gas flow geiger tube (Tracerlab TGC-14). Specific activity is defined as counts per minute per aliquot/mg of phosphorus per aliquot.

Results. Table I shows phospholipide concentration and chromatographic separation of liver phospholipides (phosphatidyl serine, phosphatidyl ethanolamine, phosphatidyl inositol, lecithins and sphingomyelins) and relative specific activity of each fraction in 8, 17, 24, 60, 145, 365 and 840 day old mice. There was an increase in concentration of μg phospholipide P/mg liver N for the 8 to 60 day old mice after which the concentration remained unchanged with age. This constancy of phospholipide phosphorus with ageing in mouse liver was observed by Schulz (9) in mice 21 to 390 days old. Williams *et al.*(10) have demonstrated a similar increase in concentration in liver of young rats 15 to 70 days old. The ratio of phospholipide P/liver N is constant in adult rats maintained on diets of different protein concentration (11) and is unaltered in animals maintained on low protein diets whether or not the liver is fatty, fibrosed, or normal(12). Concentration of phospholipides in the liver cell apparently increased only during growth. There was little change in % of total lipid phosphorus for the various phospholipide fractions with respect to age. However, the relative specific activities of each phospholipide fraction were greater in the younger mice. This observation of an increase in relative specific activities of the phospholipides is similar to that observed in the total phospholipides of the liver of 4 week old rats as compared to that of 10 weeks(13) and in 1.5 day old cultures of ascites tumor cells as compared to 6 days(14). The evidence presented supports the idea that tissue phospho-

lipides are involved in cellular growth.

Summary. 1) The influence of age upon radioactive turnover and chromatographic separation of the individual phospholipides was studied in female mice. 2) There was an increase in concentration of μg phospholipide P/mg liver N for the 8 to 60 day old mice after which the concentration remained unchanged with age. There was little change in % of total lipid phosphorus for the various phospholipide fractions with respect to age. However, the relative specific activities of each phospholipide fraction were greater in the younger mice.

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Ionic Alterations Associated with Resuscitation of the Isolated Rabbit Heart.* (26284)

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Resuscitation of the heart after chemically-induced arrest produced by potassium chloride, potassium citrate or acetylcholine has been accomplished experimentally (1,4,8), and also clinically in cardiac surgery. The isolated perfused rabbit heart may be subjected to as many as 10 successive periods of potassium ion-induced arrest with complete resuscitation in all cases (1). Furthermore, a close correlation has been found between resuscitability of the heart and temperature at which arrest was induced as well as duration of arrest. The importance of the electrolyte composition of the perfusion media employed in resuscitation procedures was stressed by all investigators. However, specific requirements for the optimum compositions are unknown. Hence the effects of cardiac arrest

per se on electrolyte content of myocardium was of interest since it might shed some light on the significance of electrolyte distribution in reinitiation of the heart beat following arrest. Therefore, the alterations in heart muscle electrolytes incident to prolonged periods of cardiac arrest were determined.

Methods. Isolated rabbit hearts were perfused by the Langendorff technic in air at an ambient room temperature of 22-24°C. Three groups of hearts were studied: 1. *Control Group* consisted of 18 isolated hearts perfused with aerated Ringer-Locke solution at 36°C for a period of 25 minutes. After this period of stabilization, 30 ml of 5% dextrose in demineralized water were perfused through the hearts to flush excess electrolytes from the vascular system. Determinations on these tissues served to establish electrolyte and water content of normal isolated heart

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TABLE I. Electrolyte and Water Content of Rabbit Heart before and after Arrest and Following Complete Resuscitation.

	Mean values in mg % $\pm$ S.E.				Water in g % (mean $\pm$ S.E.)
	Chloride	Sodium	Potassium	Calcium	
Control	413.6 $\pm$ 9.6	110.8 $\pm$ 6.7	1050.7 $\pm$ 20.6	24.3 $\pm$ 1.6	81.2 $\pm$.26
Arrested group	304.9 $\pm$ 21.3	126.3 $\pm$ 6.8	666.3 $\pm$ 20.3	19.0 $\pm$.7	80.9 $\pm$.4
P (arrested vs control)	<.001	>.15	<.001	<.01	>.45
Resuscitated group	473.5 $\pm$ 25.6	248.6 $\pm$ 14.6	549.1 $\pm$ 13.8	35.6 $\pm$ 1.8	83.6 $\pm$.2
P (resuscitated vs control)	<.02	<.001	<.001	<.001	<.001
P (resuscitated vs arrested)	<.001	<.001	<.01	<.001	<.001

muscle. 2. *Arrested Group* consisted of 14 isolated hearts perfused similarly to the control group for a period of 25 minutes. The perfusion solution was discontinued abruptly after this period and the hearts were allowed to come to complete ventricular and auricular arrest; this process required from 15 to 24 minutes. The isolated organs remained mechanically inactive for 2 hours in air at room temperature with no provisions to prevent surface drying. At that time 30 ml of 5% dextrose-demineralized water solution were perfused through the hearts; they were analyzed prior to the appearance of any mechanical contractions. These tissue samples were used for determining the effect of cardiac arrest *per se* on concentration of electrolytes of the myocardium. 3. *Resuscitated Group* consisted of 14 hearts perfused and arrested in a manner identical to the Arrested Group. After 2 hours ventricular and auricular contractions were reinitiated by perfusing with normal Ringer-Locke solution. Resuscitations were generally complete within 2-5 minutes even though several hearts displayed spontaneous irregularities in the pattern of ventricular contractions and in co-ordination of the atrial and ventricular beats. After 15 minutes of activity, 30 ml of 5% dextrose wash solution were introduced into the system and the tissues were analyzed.

For preparation of tissue samples, each of the 4 chambers of the heart was incised and rinsed thoroughly 3 times with cold demineralized water. Extraneous fat was trimmed from the myocardium and water was removed from the surfaces by blotting with gauze. Two samples of heart tissue of 4 to

8 g, each containing portions of atrium and ventricle, were used for determination of chloride and of water, sodium, potassium and calcium, respectively. Water content was determined by drying to constant weight at 105°C. The dried sample was transferred to an electric furnace and ashed at 525°C for 16 hours. The ash was dissolved in 5 ml of boiling 0.5 N hydrochloric acid and diluted to 50 ml with demineralized water. Calcium was determined by a flame photometric adaption of the Kramer-Tisdall method(2). Potassium and sodium were quantified by flame photometry using the method of Mosher *et al.*(9) which was modified by employment of standards appropriate for tissue electrolytes. Chloride content was measured on fresh tissue by the Volhard technic as used by Van Slyke and Sendroy(10).

All electrolyte concentrations were calculated on a dry weight basis.

Results. The results of quantitative determinations for sodium, potassium, calcium, chloride and water are summarized in Table I. *Water content* of the Resuscitated Group was significantly increased over both those of the Control and Arrested Groups. Water content of the Arrested Group did not differ significantly from the Control Group. *Chloride content* of the Arrested Group was significantly lower than that of Control Group. However, after resuscitation the chloride content of the hearts returned to control levels. *Sodium content* of the Arrested Group did not differ significantly from that of Control Group. On the other hand, following resuscitation, the sodium content of the hearts was twice that of either Control or Arrested

Group. *Potassium content* in the arrested hearts was significantly decreased from Control Group while potassium in the Resuscitated Group of hearts was only half of control level. A significant difference was also noted between potassium content of arrested and resuscitated hearts. Cardiac arrest resulted in a significant loss of *calcium* from heart muscle; however, following resuscitation calcium content rose markedly and exceeded both arrested and control values.

Discussion. The significant loss of potassium from the hearts following arrest and the further loss occurring after resuscitation may be responsible for the arrhythmias noted in some hearts following revival. The loss of potassium from arrested and resuscitated rabbit hearts paralleled the potassium loss observed by Harrison *et al.*(5) in congestive heart failure in man. Post-mortem examinations of ventricular muscle revealed that congestive heart failure was associated with significantly lower potassium levels (30% lower) than were noted in tissue samples from individuals with fatal non-cardiac diseases. Cummings and Clark(3) pointed out that the potassium content of infarcted cardiac muscle of dogs may be diminished to as low as 48% of that of non-infarcted tissue.

The efflux of potassium from heart muscle during cardiac arrest was accompanied by a slight sodium retention. This change could be expected since perfusion was terminated during arrest, and hence, there was no source from which sodium could be drawn to compensate for potassium outflow. After resuscitation, however, a significant influx of sodium occurred which was more than adequate to compensate the potassium loss. Cummings and Clark(3) demonstrated that sodium content of infarcted myocardial tissue averaged 71% higher than in non-infarcted tissue. It would appear that a sodium gain in excess of potassium loss is another indication of the general hypoxia persisting in the myocardium during the period of reinitiated activity after 2 hours of arrest. Leaf(7) reported a similar shift of ionic gradients between potassium and sodium in non-metabolizing heart tissue. Slices of rat heart incubated at 0°C for 50 minutes in an isotonic

medium displayed a marked gain of sodium and concomitant loss of potassium. Since maintenance of ionic gradients by tissues is considered to be an active process requiring the expenditure of energy, energy required for the transport process may no longer be sufficient when metabolism is depressed. Sodium that diffuses into the cells according to its concentration gradient can no longer be extruded and potassium can leak out of the cells in absence of active transport.

The low oncotic pressure of the perfusion medium could lead to enhanced water retention in the isolated heart; however, addition of dextran to the perfusion media failed to prevent water accumulation. The net increase in intracellular sodium may be responsible for the increased water content of resuscitated hearts. As sodium enters the cell a net gain in chloride can be expected in addition to water accumulation(7); this did in fact obtain in the resuscitated hearts. However, the role of chloride and other anionic groups, such as phosphate, bicarbonate and sulfate which were not measured in these experiments, still remains to be determined.

Tissue calcium was markedly increased in the isolated heart subsequent to resuscitation. Cummings and Clark(3) noted a 10% increase in heart muscle calcium following infarcts compared to the normal content. Thus, their results on anoxic myocardium of dogs are in general agreement with the results obtained in the isolated rabbit heart after resuscitation.

This accumulation of calcium, in combination with the excess loss of potassium after resuscitation significantly increased the tissue calcium potassium (Ca/K) ratio. This imbalance could be responsible for the varied pattern of resuscitation observed with perfusion media which would aid in sustaining the altered tissue Ca/K ratio in favor of calcium. Kolff and his associates(6) noted that resuscitation was accomplished without arrhythmias when potassium citrate was substituted for potassium chloride in elective cardiac arrest. Since citrate binds calcium, it is postulated that citrate abolishes irregularities incident to resuscitation by diminish-

ing the increased Ca/K ratio of the reperfused myocardium.

Summary. Concentrations of calcium, potassium, sodium, chloride and water have been determined following complete cardiac arrest and after cardiac resuscitation in isolated rabbit hearts. Following 2 hours of arrest both calcium and potassium were decreased significantly whereas water and sodium levels were not altered. After resuscitation following 2 hours of arrest additional potassium was lost; calcium, sodium and water content were increased significantly above control values.

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Effect of Hypothermia on Reflex Activity in the Anesthetized Dog.* (26285)

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The extent of the pressor response to exogenously increased adrenalin seems to be critically dependent on the temperature of the recipient animal. Mukherjee *et al.*(1) reported that administration of adrenalin produced a much less rise of systemic blood pressure at 29°C as compared to pressor response evoked at prehypothermic temperatures. Pressor response was further diminished at 27°C. Koella *et al.*(2) also found that magnitude of the pressor response was dependent on temperature of the animal, but at 29.9°C they showed pressor response remained unchanged from that at 37°C. At 25°C it was as little as 1/5 of that produced by the same amount of adrenalin at 37°C.

To learn more about the influence of temperature on magnitude of pressor response to exogenously increased levels of epinephrine and nor-epinephrine the following experiments were performed at normal and at reduced body temperature (28°C). Circulatory and respiratory responses to hypoxic hy-

poxia and to bilateral carotid occlusion were also studied in the same animals at normothermic and hypothermic levels to assay the reflex activity of the hypothermic animal.

Methods. Eight mongrel dogs averaging 19.6 kg were used. Dial in Urethane (Ciba), 55 mg/kg i.p., was administered before surgical procedures and was not supplemented. Observations were made at blood temperatures of 37-38°C and repeated at 27-28°C. All observations were recorded during a steady state of body temperature.

Recordings. Respiratory variables were measured from gas flow patterns, as previously described(3). Recordings were made on a Miller oscillograph. Rectal and right heart blood temperatures were measured with copper-constantin thermocouples. Systemic arterial pressure was measured from a Stat-ham pressure transducer connected to a catheter located in a femoral artery.

Epinephrine, 2 µg/kg, was injected into the superior vena cava from a catheter in an external jugular vein. Recordings were started prior to injection and continued until

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TABLE I. Percent Changes from Control of Circulatory and Respiratory Responses to Injections of Epinephrine and Nor-epinephrine, Bilateral Carotid Occlusion and Hypoxic Hypoxia in the Normothermic and Hypothermic Dog.

	Epinephrine		Nor-epinephrine		Carotid occlusion		Hypoxia	
	37°C	28°C	37°C	28°C	37°C	28°C	37°C	28°C
Heart rate	+ 3.3	-19.2	-25.6	- 23.6	+ 3.2	- 1.5	+ 11.4	+ 2.8
Syst. press.	+39.3	+49.5	+64.0	+101.9	+16.9	+ 8.9	+ 13.2	+ 6.3
Diast. "	+28.2	+25.7	+44.2	+ 87.5	+20.2	+11.9	+ 13.1	+16.9
Resp. rate	+ 2.2	-16.7	- 9.7	- 17.1	-10.9	-10.9	+120.0	+69.2
Tidal vol.	+ 7.0	+ 9.4	+ 9.8	+ 7.7	- 1.6	- 1.5	- 2.8	+12.4
Pulm. vent.	+ 9.3	+ .6	- 3.9	+ 6.1	-11.5	-11.2	+134.3	+95.9
Max. insp. flow	+22.9	+25.0	+ 2.6	+11.2	- 7.6	+ 2.0	+ 44.4	+27.2
" expir. "	+18.3	+17.8	+ .8	+ 3.8	+ 3.9	- 3.9	—	—
End tidal CO ₂	+ 7.0	+ 1.9	+ 1.8	+ 3.8	0	+ 1.9	- 30.8	-16.1

blood pressure returned to control level. Nor-epinephrine response was measured in the same manner. The common carotid arteries were clamped caudad to the carotid sinus for one minute during which time the pressor response had reached a steady state. Hypoxic hypoxia was induced by inhalation of 9% O₂ in N₂ from a Douglas bag connected to the inspiratory side of a low resistance respiratory valve. Recordings were during the 5th minute of inhalation.

Following the observations at normal body temperature the animal was partially immersed in an ice water bath. At a blood temperature of 27-28°C the procedures used as 37° were repeated.

Results and discussion. Control values just prior to drug injection, carotid occlusion or hypoxic hypoxia, were used as unity and the various respiratory and circulatory responses to these conditions were expressed as percent changes from unity for both normothermic and hypothermic conditions (Table I).

Responses to exogenously increased epinephrine and nor-epinephrine. Heart rate in the normothermic animals following injection of epinephrine was 125 as compared with a control level of 121 beats per minute. In the hypothermic animal heart rate fell from 73 to 59 beats per minute as a result of injection of epinephrine. However, in both hypothermic and normothermic animals, systolic and diastolic pressures were increased as a result of the epinephrine infusion. Systolic pressure was elevated approximately 10% more in hypothermic animals than in the normothermic condition. These results

would indicate that hypothermia potentiates the response of systolic pressure to increased (exogenously) epinephrine at 28°C. This is especially noticeable when it is considered that the rise in systolic pressure in the hypothermic animal is greater than that in the normothermic animal even though heart rate is decreased in the hypothermic animal during blood pressure rise. Respiratory changes as a result of epinephrine injection were approximately the same under the 2 body temperatures studied. Respiratory rate was decreased from an average of 13.2 breaths per min to 11.0 in the hypothermic animals during the epinephrine-caused blood pressure rise. This is probably related to the interplay between blood pressure rise and respiratory rate changes usually seen as a result of the elevated pressure. End tidal CO₂ was increased from 5.7 to 6.1% in the normothermic animals and from 5.2 to 5.3% in the hypothermic animals following injection of epinephrine.

Quantitatively, 2 major differences were seen between responses to epinephrine and nor-epinephrine. Whereas little change occurred in heart rate in the normothermic animals following epinephrine injection the same dosage of nor-epinephrine decreased the average heart rate from 125 to 93 beats per minute. Approximately the same percentage decrease in heart rate was observed at reduced body temperature. Also, in the normothermic animals nor-epinephrine increased systolic pressure 64% but in the hypothermic state an increase 102% was seen. These increases were of a higher order of magnitude than were observed with epinephrine. Again

(as with epinephrine) the greater increase of systolic pressure was observed in hypothermia in presence of a slower heart rate. These results indicate that the hypothermic animal is more sensitive to exogenously increased nor-epinephrine than is the normothermic animal.

The disagreement between the results of epinephrine administration reported here and those of Koella *et al.*(2) and Mukherjee *et al.*(1) may be related to depth of anesthesia and/or to dosage of the hormones administered. Doses of adrenaline administered by Mukherjee *et al.* varied from 100-200 μ g in cats anesthetized (i.m.) with urethane in dosage of 1.5 to 1.8 g/kg. Koella and colleagues administered adrenalin at a dosage of 20 gamma but their animals (cats) were anesthetized with Dial urethane i.p., 25 mg/kg body weight, and immobilized with intocostrin so that artificial respiration was necessary. Our experiments are more closely related to those of Koella *et al.* with respect to dosage of adrenalin used but differ markedly in depth of anesthesia. However, we agree with these two groups on the findings that cardiac slowing induced by the pressor response is more marked in the hypothermic condition than in the normothermic.

Effect of hypothermia on carotid sinus reflex. Heart rate was essentially unchanged as a result of this maneuver at both normal and reduced body temperatures. Systolic pressure was increased from 130 to 152 mm Hg at normal body temperature and from 112 to 122 mm Hg at 27°C, increases of 17% and 9% respectively. Diastolic pressure was affected in essentially the same manner. Reduction in body temperature did not grossly change respiratory responses except for maximum inspiratory flow rate which decreased 7.6% in normothermia and increased 2% at 28°C as a result of carotid occlusion. These data show that the hypothermic animal retains reflex activity when body temperature is reduced 9-10°C. These findings are in agreement with Nashat and Neil(4) who elicited carotid sinus reflexes by raising the static pressure in one isolated carotid bifurcation. They found that respiratory and cir-

culatory reflex responses were invariably present in cats at 26°C although the reflex fall of blood pressure produced by sinus hypertension was of less extent than at normal temperatures.

Effect of hypothermia on respiratory and cardiovascular responses to hypoxic hypoxia. Heart rate was increased 11% during the 5th min of exposure to the low oxygen gas mixture at normal body temperature: at reduced body temperature an increase of 3% was recorded. Systolic pressure increased 13.2% and diastolic pressure 13.1% at 37° as a result of the hypoxic hypoxia. These values were 6.3% and 16.9%, respectively at 28°C.

More pronounced differences between the warm and cold conditions occurred in respiratory responses to hypoxic hypoxia. Respiratory rate was more than doubled (37.6 to 82.7 breaths per min) during normothermia but increased only 69% (12.0 to 20.3) in hypothermia. Tidal volumes were relatively unchanged at the 2 levels of body temperature so that pulmonary minute volume was not as greatly elevated in hypothermia as at normal temperature. Maximum inspiratory air flow rates were different at the different body temperatures and are related to the changes in respiratory rates. The normothermic animal hyperventilates relatively more than the hypothermic animal exposed to hypoxic hypoxia as evidenced by the differences in end tidal CO₂. Hypoxic hypoxia produced a decrease of 31% in end tidal CO₂ at normal body temperatures whereas a fall of 16% was recorded at the lowered body temperature. These results indicate that the hypothermic animal maintains the capacity to respond to hypoxic hypoxia *via* stimulation of the peripheral chemoreceptors.

Kilmore and Chase(5) reported the response of arterial pressure to inhalation of 15% O₂ was essentially the same under normothermic and hypothermic conditions in dogs. Systemic circulatory and respiratory responses of cats at 26°C to anoxia (produced by inhalation of 10% O₂ in N₂) were reported as very feeble by Nashat and Neil (4). The differences in results obtained by

the various groups again may be attributed to depth of anesthesia or perhaps an inherent difference between hypothermic responses of cats and dogs.

Summary. Evidence is presented which indicates that pressor response to injections ($2 \mu\text{g/kg}$) of epinephrine and nor-epinephrine is potentiated in the anesthetized dog at blood temperatures of $27-28^\circ\text{C}$. The augmented response to nor-epinephrine was greater than that for epinephrine. Evaluation of respiratory and circulatory responses to bilateral carotid occlusion and hypoxic hypoxia indicates that the hypothermic dog re-

tains reflex activity but magnitude of the responses is less than that at normal body temperature.

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Plasma Free Fatty Acids and the Rare-Earth Fatty Liver.* (26286)

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Some of the lanthanide elements when injected intravenously into rats and other species can produce a fatty liver cycle that is affected by certain endocrines(1,2). The influence of endocrines on release of free fatty acids (FFA) from adipose tissue and the implied significance of FFA in lipid transport (3-6) suggested to us that mobilized extrahepatic lipids might be the source of at least part of the increased liver glyceride level caused by cerium. The experiments described here have tested this possibility; the data demonstrate that cerium does cause an increase in free fatty acids of the plasma and that substances which bind cerium or aggregate it prevent the FFA response and the fatty liver.

Method. Female Carworth-Farms-Nelson (CFN) albino rats weighing 150-200 g were used in all of the experiments except for one group of 7-male CFN rats (136-160 g) and another group of Charles River hypophysectomized female rats (139-170 g). Cerous chloride[†] (2 mg Ce/kg) was injected intravenously into the tail vein. All animals were

maintained on a Dietrich and Gambrill[‡] laboratory chow before the cerium injection; some animals were fasted 24 hr before death and others were fed *ad libitum*. Water was available at all times. At 24, 30, 48, and 72 hr after cerium injection, the animals were anesthetized with ether and blood was drawn in a heparinized syringe from the abdominal aorta. The plasma free fatty acids were extracted and determined in duplicate by the procedure of Dole(3). An Ultra Micro-Buret, model 200, obtained from Scientific Industries, Inc., was used for all titrations. In a few rats, glucose was measured(7) in serum obtained from blood after decapitation.

Cerium in combination with versene, albumin, adenosine triphosphate, plasma, phosphate, hydroxide, adenine, adenosine, cytidine, citric acid, and heparin was injected into rats to see whether these compounds protected the animal from the fatty liver degeneration. The method for extraction and measurement of total liver lipids has been described(1).

Results and discussion. A significant in-

* Under contract with U. S. Atomic Energy Commission.

[†] Cerous chloride, Fisher, C-252, dissolved in saline.

[‡] Dietrich & Gambrill, Inc., Laboratory Animal Food Division, Frederick, Md.

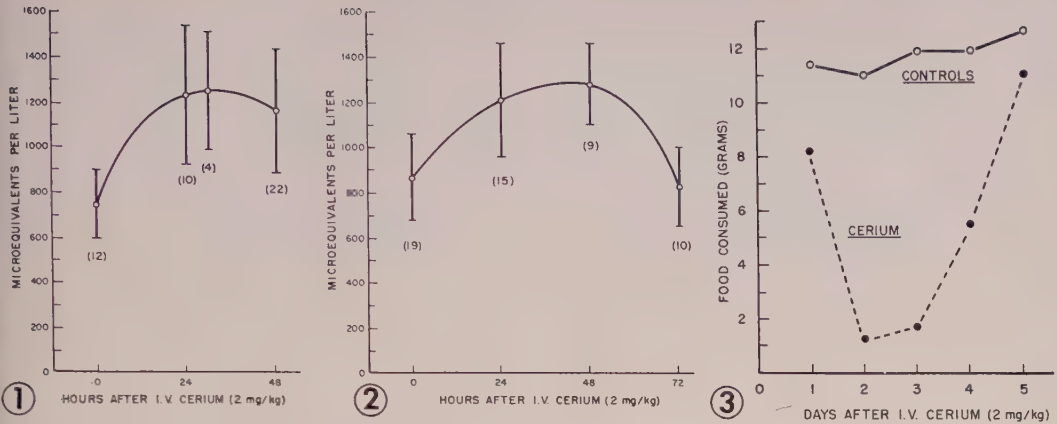


FIG. 1. Effect of cerium on plasma free fatty acids in female rats fed *ad libitum*. Vertical bars represent stand. dev. No. of animals are shown in parentheses. The probability for chance occurrence was less than 0.001 when the test of significance was applied to the difference between the mean of control group and means of 24, 30, and 48 hr post-cerium groups.

FIG. 2. Effect of cerium on plasma free fatty acids in female rats fasted 24 hr before sacrifice. Vertical bars represent stand. dev. No. of animals shown in parentheses. The probability for chance occurrence was less than 0.001 when the test of significance was applied to the difference between means of control group and means of 24 and 48 hr post-cerium groups.

FIG. 3. Effect of cerium on food consumption. Each group consisted of 5 rats.

crease in plasma free fatty acids precedes the development of fatty livers in rats injected intravenously with cerium (Fig. 1). The high levels of titratable acid at 24 hr after cerium administration cannot be explained by the reduced food intake which occurs during this period (8 g, as seen in Fig. 3), since the effect of cerium on FFA is also observed in fasted rats (Fig. 2); furthermore, plasma

TABLE I. Effect of Various Treatments on Plasma Free Fatty Acids in CFN Rats Injected with Cerium.

Treatment	No. of rats	Free fatty acids	
		$\mu\text{eq/l}$	Range
Controls	12*	754	(512-1025)
24 hr cerium	10*	1237	(867-1900)
Versene-cerium	4*	613	(537- 713)
Cerium, followed by versene	4*	1004	(900-1160)
Reduced food intake (6 g per day)	5	681	(549- 750)
Controls (24 hr fast)	3	976	(855-1217)
" (male)	4*	641	(523- 834)
Cerium—24 hr (male)	3*	666	(525- 750)
Cerium hydroxide	4*	515	(435- 563)
Hypophysectomized control (24 hr fast)	4	709	(665- 771)
Hypophysectomized, 24 hr cerium (24 hr fast)	5	782	(650- 938)

* Fed *ad libitum*. All animals were female, unless otherwise indicated in Table.

TABLE II. Effect of Various Compounds on Total Liver Lipids 24 Hr after an Intravenous Injection of Cerium.

Compound admin. with cerium, I.V.	Molar ratio: Compound/Ce	Liver lipids, % wet tissue
None	—	13.92, 14.87
Versene	5.65	4.60, 6.87
Albumin	.03	10.01, 11.66
ATP	.60	4.39, 5.53
Phosphate	2.26	5.88, 5.98
Hydroxide	excess	5.29, 6.14
Adenine	1.04	5.51, 7.19
Adenosine	1.00	10.67, 11.95
Cytidine	1.00	9.90, 10.71
Citric acid	5.00	10.19, 12.85
Plasma	—	6.40, 7.26
Heparin*	—	14.94, 16.59

* 1 mg at 15 min. before and 6, 24, 30 and 47 hr after cerium inj. All other compounds were administered as a single intrav. inj. in combination with cerium.

FFA levels are not significantly altered in control rats fed a restricted diet (6 g, as seen in Table I).

Conditions that prevent the induction of the cerium fatty liver also prevent the free fatty acid response; *e.g.*, use of male or hypophysectomized rats, versene binding, and particle formation *via* hydroxide. Table II shows the protective action of certain compounds mixed with cerium before injection in development of this fatty liver infiltration. All the substances that either bound with

TABLE III. Effect of Cerium (2 mg/kg, I.V.) on Serum Glucose.

Treatment	No. of rats	Glucose (range), mg/100 ml	Liver fat
Control (<i>ad libitum</i>)	3	172 (161-178)	—
Control (24 hr fast)	6	144 (137-164)	—
4 hr cerium (<i>ad libitum</i>)	4	141 (134-145)	—
24 hr cerium (24 hr fast)	4	91 (76-126)	+
48 hr cerium (24 hr fast)	4	88 (74-95)	++++
<i>Idem</i> , alloxan diabetic rats)	3	308 (205-468)	—

cerium (versene, plasma proteins) or produced particles (phosphate, hydroxide, ATP) also prevented the usual increase in liver lipids of animals killed 48 hr after an intravenous cerium dose (2 mg/kg). The results obtained with adenine or adenosine are difficult to explain. It can be seen from Table I that the cerium must be reacted with the substance in question before injection, since the fatty liver and the FFA response will not be prevented if versene, for example, is administered immediately before or after cerium injection. Preliminary experiments in our laboratories, in which versene has been infused continuously in dogs attached to an artificial kidney with hopes of speeding up excretion of cerium, have also demonstrated that the biological binding of cerium is quite tenacious.

The inverse relationship of blood glucose and FFA levels that occurs after certain hormonal stimuli(3,6) also exists with the cerium-induced free fatty acid response, *i.e.*, a decrease in serum glucose occurs as early as 4 hr after administration of cerium (Table III). A similar effect of rare earths on blood sugar is described in a review by Trapmann (8). Because of the involvement of carbohydrate, the effect of insulin deprivation was tested, and the alloxan diabetic rat, like the hypophysectomized animal, will not develop

the cerium-induced fatty infiltration (Table III). The fact that serum glucose decreases at a time when plasma FFA levels from cerium-treated rats (0.5 h, 728 μ Eq/L and 4 hr, 759 μ Eq/L) are in the same range as controls, indicates that carbohydrate metabolism is interrupted before the lipid disorder occurs.

The consistent occurrence of elevated free fatty acids during accumulation of total liver lipids suggests that mobilization from other sites is an important factor in the mechanism of fatty infiltration due to cerium. Other experiments, including measurements of distribution of total body lipids and changes in their composition by gas chromatographic analyses, have been initiated to evaluate this concept. A depressed mitochondrial oxidation of lipids (octanoic acid) is also a contributing factor in their abnormal accumulation in liver after cerium administration(9).

Summary. 1. Cerium (2 mg/kg) causes an early decrease in serum glucose and a later significant increase in plasma free fatty acids followed by fatty degeneration of the liver. 2. Conditions that prevent the free fatty acid and fatty liver responses are (a) administration of the cerium as a bound complex or particle, and (b) use of hypophysectomized, diabetic, or male rats.

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Effect of Dilantin Sodium Analogues on Cell Proliferation in Tissue Culture.* (26287)

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Previous investigations in this laboratory have shown clearly the stimulatory effect of sodium diphenylhydantoinate (Dilantin Sodium)[†] on proliferation of human gingival fibroblast-like cells in tissue culture(1). It has been suggested that this stimulation of cell proliferation is related to the well-recognized phenomenon of gingival hyperplasia occurring in patients receiving Dilantin Sodium for treatment of epilepsy(1), to the increased rate of healing of gingival wounds in non-epileptic human patients receiving Dilantin(2), and to the increased tensile strength of healing skin wounds in rats receiving Dilantin(3). This finding of accelerated wound healing as judged by increased tensile strength in Dilantin-treated animals as well as animals given 3 ethyl, 5 phenylhydantoin has been confirmed by Kelln and Gorlin(4). Fibroblastic proliferation with increased collagenization would explain these *in vivo* phenomena and could be accounted for by the *in vitro* findings.

In seeking an explanation for the basic mechanism underlying this unusual cell response, a series of chemical analogues of Dilantin Sodium as well as a group of structurally related compounds have been tested for a possible similar stimulatory ability.

Materials and methods. The line of human gingival fibroblasts (E.D.) used in previously reported studies(1) was lost shortly after completion of those studies both in our laboratory and the laboratory from which it was originally obtained (Dr. Donald E. Flieder, St. Louis Univ. School of Dentistry). For this reason, another cell line maintained in our laboratory was tested in these studies with various compounds for effects on cell proliferation. This particular cell line was

a human gingival fibroblast-like cell isolated and cultured from gingival tissue removed by surgical excision from a 12-year-old female patient with the condition known as fibromatosis gingivae.[‡] This cell line was in its 21st transfer at time of initiation of these studies, having been maintained continuously for approximately 4 months.

The medium used for growth of this fibroblast-like cell consisted of: 30 ml calf serum, 4.5 ml Earle's basal salt solution (20× conc.), 3 ml vitamin mixture (100× conc.)[§], 3 ml amino acid mixture (100× conc.)[§], 3 ml glutamine solution (200 mM, 100× conc.)[§], and 50 ml triple distilled water with 100 units penicillin, 200 µg streptomycin and 50 units Mycostatin (Squibb) per ml of medium. This medium was adjusted to pH 7.0 with sodium bicarbonate.

In testing the various compounds to be reported here, actively growing cultures of fibroblast-like cells were trypsinized (0.25% solution of trypsin) for 15 minutes at 37°C, centrifuged at low speed for 20 minutes, washed in single strength Earle's BSS and centrifuged once more. The cells were then resuspended in the nutrient medium and to each bottle containing 80 ml of medium was added 2 ml of an aqueous solution of Dilantin Sodium, the various analogues or structurally related compounds each prepared at a concentration of 200 µg per ml of water.

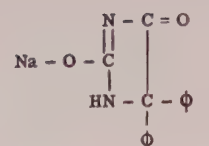
After obtaining viable cell counts of control and experimental suspensions and adjusting initial suspensions to pH 7.0, 4 ml portions of each of the control and experimental suspensions of cells were dispensed into a series of tissue culture tubes and the tubes incubated at 37°C in a stationary position. At the various periods indicated in the tables, cells of 3 tubes picked at random from each

* Supported by grant from Nat. Inst. of Dental Research, U.S.P.H.S.

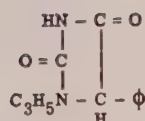
† Parke, Davis & Co., Detroit, Mich.

‡ Courtesy of Dr. Henry M. Swenson.

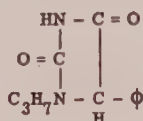
§ Microbiological Associates, Bethesda, Md.



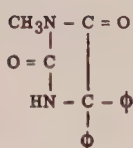
Sodium, 5, 5 diphenyl hydantoinate (Dilantin)



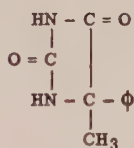
1 allyl, 5 phenyl hydantoinate



1 propyl, 5 phenyl hydantoinate



3 methyl, 5, 5 diphenyl hydantoinate



5 methyl, 5 phenyl hydantoinate

FIG. 1.

group were suspended by addition of 4 ml of 0.25% trypsin solution to each, and viable cells counted in a hemocytometer after staining with trypan blue (0.5% solution).

The chemical analogues of Dilantin Sodium tested in this study were: 1) 1 allyl, 5 phenyl hydantoinate; 2) 1 propyl, 5 phenyl hydantoinate; 3) 5 methyl, 5 phenyl hydantoinate and 4) 3 methyl, 5, 5 diphenyl hydantoinate,|| while the structurally related compounds were: 1) barbitol sodium, 2) urea, 3) uric acid, 4) hydantoin, 5) allantoin and 6) alloxan (Fig. 1 and 2).

Results. The data shown in both Tables I and II clearly confirm the remarkable stimulatory effects of Dilantin Sodium on proliferation of human gingival fibroblast-like cells in tissue culture. In most cases, the counts of cells exposed to Dilantin Sodium were approximately double those of control tubes by the 4th or 5th days of incubation. Each value expressed in Tables I and II repre-

sents the average count on 3 tubes with occasional exceptions. Two of the Dilantin analogues (1 allyl, 5 phenyl hydantoinate and 5 methyl, 5 phenyl hydantoinate) stimulated cell proliferation to the same significant degree as Dilantin Sodium (Table I).

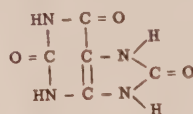
TABLE I. Effect of Dilantin Analogues on Proliferation of Human Fibroblast-Like Cells in Tissue Culture.

	Mean cell count (per ml of medium) $\times 10^5$					p (2-7 day intervals)
	0	1	2	4	7	
Control	1.01	1.18	1.65	2.23	4.00	—
Dilantin	1.01	1.20	3.03	4.26	6.04	<.01
Analogue A*	1.01	1.18	3.10	4.22	6.02	<.01
" B*	1.00	1.17	1.66	2.36	4.06	N.S.

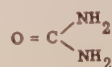
	Mean cell count (per ml of medium) $\times 10^5$				p (2-5 day intervals)
	0	1	2	5	
Control	1.03	1.28	1.51	3.00	—
Dilantin	1.07	1.26	2.95	4.58	<.01
Analogue C*	1.04	1.27	3.00	4.82	<.01
" D*	1.05	1.26	1.50	2.90	N.S.

* Analogue A, 1 allyl, 5 phenyl hydantoinate; Analogue B, 1 propyl, 5 phenyl hydantoinate; Analogue C, 5 methyl, 5 phenyl hydantoinate; Analogue D, 3 methyl, 5, 5 diphenyl hydantoinate.

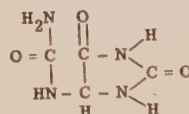
The other two very closely related compounds (1 propyl, 5 phenyl hydantoinate and 3 methyl, 5, 5 diphenyl hydantoinate) were completely ineffective in producing a stimulatory response, at the periods shown, similar to



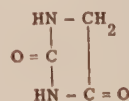
Uric acid



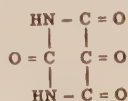
Urea



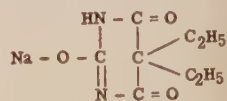
Allantoin



Hydantoin



Alloxan



Barbital sodium

FIG. 2.

|| The 4 chemical analogues were courteously supplied by Parke, Davis & Co., Detroit, Mich.

TABLE II. Effect of Compounds Structurally Related to Dilantin on Proliferation of Human Fibroblast-Like Cells in Tissue Culture.

	Mean cell count (per ml of medium) $\times 10^5$		p (4 day interval)
	0	4	
Control	.96	2.02	—
Dilantin Na (.0007M)	.97	4.19	<.01
Barbital Na (.0010M)	.98	2.03	N.S.
Hydantoin (.0019M)	.98	2.02	"
Allantoin (.0013M)	.98	2.08	"
Uric acid (.0012M)	.98	2.02	"
Urea (.0033M)	.99	2.03	"
Alloxan (.0014M)	.98	2.02	"

Dilantin Sodium or the other 2 chemical analogues.

None of the structurally related compounds (barbital sodium, urea, uric acid, alloxan, allantoin or hydantoin) produced a stimulatory effect similar to that of Dilantin Sodium (Table II). At the 4-day interval, found adequate through numerous studies in this laboratory for evaluation of cell-stimulation effects, cell counts in all tubes except Dilantin Sodium did not differ significantly from those of control tubes.

Discussion. The studies here obviously confirm the cell stimulatory effect of Dilantin Sodium. However, they fail to clarify that particular portion of the hydantoin molecule or side chain or grouping of side chains responsible for this phenomenon. The

phenomenon is apparently unrelated to the basic hydantoin structure, presence of phenyl groups, or presence or absence of various groups at the 1 or 3 position. It is interesting to note that, while analogues B and D and all the structurally related compounds were ineffective in stimulating cell growth, there was no evidence of growth inhibition or cytotoxicity.

Summary. Several chemical analogues and chemically related compounds of Dilantin Sodium have been used to study and clarify the cell stimulatory effects of this compound. Two analogues (1 allyl, 5 phenyl hydantoinate and 5 methyl, 5 phenyl hydantoinate) induce stimulation of fibroblast-like cell proliferation while 2 other analogues (1 propyl, 5 phenyl hydantoinate and 3 methyl, 5,5 diphenyl hydantoinate) were completely ineffective. None of the other structurally related compounds tested (barbital sodium, urea, uric acid, alloxan, allantoin or hydantoin) produced any stimulation of cell proliferation.

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A Study of Bentonite Flocculation Test for Measurement of Type-Specific Streptococcal Antibodies.* (26288)

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A reliable and sensitive technic for determining type-specific anti-streptococcal antibodies in the blood of patients recovering from Group A streptococcal infections would help greatly in investigating epidemiological and clinical problems associated with such

infections and their sequelae. The mouse-protection test(1), which is generally accepted as the ultimate basis for type classification of Group A hemolytic streptococci and for detection and measurement of type-specific protective antibody, has the disadvantage that it requires strains of high mouse virulence (which are infrequent among streptococci freshly isolated from human sources),

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and a high antibody content in the human serum. Furthermore, large amounts of sera are necessary to carry out the tests.

The bacteriostatic test(2,3) is based on the observation that the union of homologous antibody with M antigen on the surface of the bacterial cells increases susceptibility of these cells to phagocytosis by human leukocytes. The inhibitory effect of antibody is most marked with strains containing the largest amount of M antigen. Cross-reactions between some types such as 2, 13, and 48 have been observed(4). This test requires fresh "normal" human whole blood, drawn not more than 2 hours prior to the test proper; blood from laboratory animals cannot replace the "normal" human blood(5).

Stollerman and Ekstedt(6) during their studies on the bactericidal system(2) for detection of type-specific antibody, found a number of strains which grew in extremely long chains (approximately 50 to 100 cocci per unit as compared to chains of 4 to 12 observed in the control), in the presence of homologous antiserum. Their data indicate that formation of long chains in liquid media by the strains of streptococci studied is dependent in part upon the interaction of type-specific antibody and the M protein of the cells.

The complement-fixation test has been employed by some investigators in measurement of type-specific antibody(7), but repeated absorption of the antisera with strains of heterologous types is necessary to minimize cross-reactions.

Boyden(8) showed that treatment of sheep erythrocytes with solutions of tannic acid and insulin rendered them capable of adsorbing proteins, including Group A streptococcal M protein, from solutions in normal saline; the cells subsequently washed in normal saline were found to be agglutinable by the corresponding antiprotein sera. This hemagglutination test, when used to measure type-specific antistreptococcal antibody, also requires absorption of serum with heat-killed Group A streptococci of heterologous types(9).

Since the major difficulty in the latter 2 procedures is the finding that crude antigenic

preparations derived from streptococci react not only with homologous type-specific antibodies but also with antibodies stimulated by immunization with heterologous serologic types, and since in any given patient's serum antibodies to multiple Group A streptococcal types may occur, then the specificity of any observed reaction will necessarily depend upon the specificity of the antigen used. It should be noted that preparation of highly purified type-specific antigen is extremely difficult and is regarded by some workers as impossible of achievement with the methods currently used. A second approach to this problem is in the use of absorption to remove heterologous antibody from the patient's serum before testing. While this is possible to achieve, it too is a laborious procedure and one difficult to apply to large numbers of serum specimens.

Bozicevich(10) demonstrated that bentonite[†] suspensions sensitized with an acid-soluble protein fraction extracted from *Trichina* larvae provide 95% or greater accuracy in serological diagnosis of trichinosis. Besides being highly accurate, the test can be completed within 20 minutes in any ordinary laboratory. Another advantage is that the stock bentonite suspension can be kept at room temperature for as long as 4 months without losing its potency, while antigen-coated-bentonite suspension can be stored at 4°C for 2 months(10). He applied this technic successfully to the serological diagnosis of rheumatoid arthritis(11). Both of his tests have withstood intensive evaluations by other investigators(12,13).

There have been no publications on the feasibility of this method for detection of type-specific anti-streptococcal antibodies in sera of infected humans or of immunized laboratory animals; to investigate its possible usefulness the present study was undertaken.

Materials and methods. A. *Bacterial strains and antisera.* The streptococcal strains employed and production of the rabbit antisera have been described(14). B. *Preparation of Group A streptococcal crude M protein.* The crude M protein was ex-

[†] Bentonite is a montmorillonite clay obtained from Fisher Scientific Co., Fair Lawn, N. J.

tracted from each strain with hot hydrochloric acid according to the method of Lancefield(15) with the following modifications: (a) the final extract was precipitated twice with alcohol after it was demonstrated by the capillary precipitin test of Swift, Wilson, and Lancefield(16) that there was no group-specific C substance in the extract; (b) the crude M antigen in buffered normal saline was preserved with 1-5,000 Merthiolate and stored at 4°C. C. *Preparation of Group A streptococcal Somatic Polysaccharide*. Strain S-94 was grown in trypticase-soy broth at 37°C for 18 hours and the group-specific polysaccharide was extracted from the cells by Fuller's formamide method (17). The polysaccharide extract was stored at 4°C. D. *Preparation of stock bentonite suspension*. The stock bentonite suspension was prepared according to the procedure of Bozicevich(10). E. *Preparation of test antigen*. Bentonite suspensions with adsorbed M antigen will hereafter be referred to as "test antigen." Absorption of M antigen onto the bentonite particles was carried out in the following manner:

- 1) A 2.5 ml portion of M antigen solution was added to 5.0 ml of stock bentonite suspension in a 40.0 ml conical graduated centrifuge tube.
- 2) The tube was left standing vertically in the refrigerator overnight—approximately 15 hours.
- 3) The mixture was removed, shaken, and 0.625 ml of 0.1% aqueous methylene blue solution was added to it.
- 4) It was again shaken gently by hand for 3 to 5 minutes.
- 5) 10 ml of normal saline were added, and the mixture was shaken thoroughly for one minute.
- 6) The mixture was centrifuged at 1,600 rpm for 15 minutes, using a size 2 International Centrifuge, with No. 240 head.
- 7) The supernate was discarded; the sediment was suspended in 15.0 ml of normal saline and centrifuged at 1,600 rpm for 15 minutes.
- 8) The resultant supernate was discarded; the sediment was resuspended in 4.0 ml of 0.05M phosphate buffer at pH 8.5.
- 9) Five tenths ml of normal rabbit serum (pre-immunization serum) was added to prevent spontaneous flocculation.
- 10) The suspension (test antigen) was stored

at 4°C until used. F. *Bentonite flocculation test*. The test is a modification of the method used in diagnosis of trichinosis(10). It was carried out as follows: 1) Two-fold serum dilutions were prepared with 0.05M phosphate buffer at pH 8.5. 2) With a clean glass pipette delivering 30 drops per ml, 2 drops of each dilution were put into each ring of a 10-ring glass slide, using a clean pipette for each dilution. 3) The test antigen was removed from storage at 4°C and allowed to reach room temperature before use. It was shaken to obtain a homogeneous suspension, then with a clean glass pipette delivering 30 drops per ml, one drop was added to the center of each ring containing the diluted serums. 4) The slide was then placed on a Clay-Adams horizontal rotator operated at 100-120 rotations per minute for 15 minutes, after which readings were made immediately, to avoid drying, under a microscope (60 ×).

Positive and negative serum controls were included in each series of tests, together with a suspension control consisting of sensitized particles and buffer only.

Results and discussion. Contrary to expectation from the report of Bozicevich(10) it was found that spontaneous flocculation occurred when normal saline buffered at pH 7.2 was used to suspend the antigen-coated bentonite particles. Use of Tween 80 in concentrations even greater than the recommended 1% did not prevent this. However, in presence of 0.05 M acetate buffer, pH 2.6, or 0.05 M phosphate buffer at pH 7.3 or 8.5, spontaneous flocculation did not occur if normal rabbit serum was included, as described in the previous section; the M protein-coated bentonite remained in suspension without loss of potency for as long as 6 weeks.

In bentonite flocculation tests involving 3 types of Group A streptococci (types 12, 14, 36) (Tables I, II, and III), the titers were considerably lower than those obtained by the hemagglutination technic(9). The results thus suggest that a fairly high antibody level in the test serum may be required for bentonite flocculation, which would somewhat lessen the value of the test for diagnostic use. It is of interest to note that the

TABLE I. Flocculation Reactions: Test Antigen M (Lot #8) from Type 14 Strain S-23 *vs* Homologous and Heterologous Antisera.

Rabbit No.	Dilutions	1-2	1-4	1-8	1-16	1-32	1-64	1-128	1-256
	Buffer	0	—	—	—	—	—	—	—
73	Pre-imm. serum	0	0	0	0	0	0	0	0
	Antisera								
16	Homologous	4+*	4+	4+	4+	3+	2+	0	0
54	"	4+	4+	4+	2-3+	2+	1+	0	0
72	"	4+	4+	4+	2+	0	0	0	0
16	"	4+	4+	4+	4+	3+	2+	1+	0
76	Anti-type 36	3+	1+	0	0	0	0	0	0
77	<i>Idem</i>	4+	0	0	0	0	0	0	0
51	Anti-type 12	0	0	0	0	0	0	0	0
9	<i>Idem</i>	0	0	0	0	0	0	0	0

* 4+ indicates agglutination of all particles.

TABLE II. Flocculation Reactions: Test Antigen M (Lot #10) from Type 36 Strain S-94 *vs* Homologous and Heterologous Antisera.

Rabbit No.	Dilutions	1-2	1-4	1-8	1-16	1-32	1-64	1-128	1-256
	Buffer	0	—	—	—	—	—	—	—
77	Pre-imm. serum	0	0	0	0	0	0	0	0
	Antisera								
77	Homologous	4+	4+	4+	3+	1+	0	0	0
76	"	4+	4+	4+	3+	1+	0	0	0
74	"	4+	4+	4+	3+	2+	0	0	0
73	"	3+	3+	2-3+	1+	0	0	0	0
9	Anti-type 12	0	0	0	0	0	0	—	—
51	<i>Idem</i>	0	0	0	0	0	0	—	—
52	"	0	0	0	0	0	0	—	—
16	Anti-type 14	0	0	0	0	0	0	—	—
54	<i>Idem</i>	0	0	0	0	0	0	—	—

TABLE III. Flocculation Reactions: Test Antigen M (Lot #1) from Type 12 Strain Bailey *vs* Homologous and Heterologous Antisera.

Rabbit No.	Dilutions	1-2	1-4	1-8	1-16	1-32	1-64	1-128	1-256
	Buffer	0	—	—	—	—	—	—	—
9	Pre-imm. serum	0	0	0	0	0	0	0	0
	Antisera								
9	Homologous	4+	4+	3+	2+	0	0	0	0
51	"	4+	4+	4+	1+	0	0	0	0
52	"	4+	4+	4+	2+	0	0	0	0
16	Anti-type 14	0	0	0	0	0	0	0	—
72	<i>Idem</i>	0	0	0	0	0	0	0	—
73	Anti-type 36	0	0	0	0	0	0	0	—
74	<i>Idem</i>	0	0	0	0	0	0	0	—

cross-reaction between types 36 and 14 M antigens was not reciprocal. M antigen extracted from strain S-94 (type 36) failed repeatedly to react with heterologous anti-S-23 (type 14) antisera. However, in these cross-reactions, even though they were either 3+ or 4+ in degree, the clumps were small as compared to the large, compact clumps seen in the homologous reactions at low dilutions.

A mixture of C substance and M antigen was obtained by hot acid extraction(18) from a culture of strain S-23 (type 14). Capillary precipitin tests using both group-specific and type-specific antisera were performed. A 4+ reaction was obtained in each case. Sensitization of stock bentonite was carried out in the usual way but with substitution of this mixed antigen for the ethyl alcohol-precipi-

tated M antigen. Flocculation tests using both type-specific and group-specific antisera were negative with all of the test serum dilutions. Why no flocculation occurred, especially in presence of the type-specific antisera, is not known. Partially purified M protein obtained from such a preparation was normally reactive with homologous antisera when adsorbed to bentonite particles.

There appear to have been no publications in the North American literature on adsorption of polysaccharide antigens by bentonite. C substance, obtained by the hot formamide extraction method of Fuller(17) was used in an attempt to sensitize bentonite particles by the standard procedure. In the presence of group-specific antiserum there was no flocculation.

Summary. A flocculation test for detection and measurement of type-specific antibody to Group A streptococci has been evaluated. A single strain of each of 3 serological types of streptococcus was employed. When a suspension of bentonite particles between 1.0 and 1.5 μ in size was exposed to an unadsorbed homologous rabbit antiserum after being coated with partially purified M protein antigen, flocculation was obtained. The highest homologous flocculation titer encountered was 1-32. A weak non-reciprocal cross-reaction between types 36 and 14 M antigens was found: while M antigen of strain S-94 (type 36) did not react with anti-S-23 (type 14) antisera, M protein of strain S-23 exhibited some cross-reactivity with anti-S-94 antisera. The test antigen could be stored at 4°C for at least 6 weeks without loss of potency. No group-specific or type-specific flocculation occurred when the test antigen was prepared by exposing the bentonite particles to a crude antigen mixture, obtained by hot HCl extraction of streptococcal cells, which was shown

by precipitin tests to contain large amounts of both group-specific and type-specific antigens. Partially purified M protein obtained from such a mixture, however, could be used to sensitize particles, which were then reactive with antisera. No flocculation was observed when bentonite particles treated with group-specific polysaccharide solution were exposed to homologous group-specific antiserum.

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Demonstration of Venezuelan Equine Encephalomyelitis in Tissue Culture By Immunofluorescence. (26289)

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With the advent of cell culture technics the possibilities of clinical detection of virus diseases are greatly enhanced. To accomplish this, however, it is necessary to obtain maximal cytopathogenic effect (CPE), numerous passages to assure that the CPE is due to a specific virus, and finally, specific identification by neutralization or other serologic tests. The introduction of immunofluorescent technics has presented a possibility of detecting virus in tissue culture cell lines prior to specific changes in the infected cell. A model virus system was studied. Venezuelan equine encephalomyelitis (VEE) was selected as it can be studied in all phases of cell culture, from its initial inoculation to marked CPE.

Materials and methods. The virus strain was one which had been adapted to a guinea pig heart cell line (GPHC) by Berge *et al.* (1). The starting strain was an egg-adapted Trinidad VEE isolate. During 80 passages in GPHC there had been changes in virulence for various laboratory animals without known alterations in serologic activity. The cells were maintained in Eagle's media(2) with 20% horse serum and antibiotics added. Infected and noninfected cells were grown on cover slips, in a Petri dish, at 37°C under 5% carbon dioxide.

The antiserum, provided by Gochenour(3), was from a burro (*Equus asinus*) that was bled 2 weeks after an intramuscular inoculation of viable VEE. The serum gave a hemagglutinating titer of 1/640 and a complement-fixing titer of 1/520.

The serum was divided into 3 portions: (a) whole serum without any treatment (WS); (b) whole serum in which the globulin fraction was precipitated with methanol (4) and reconstituted to four-fifths volume (GP); (c) whole serum absorbed by adding

1 g of tissue culture cells per 5 ml and incubating 30 minutes at 37°C with intermittent shaking, then adding 50 mg per cc of acetone-dried rabbit powder(5) and shaking at room temperature for 1½ hours, finally centrifuging in the cold at 15,000 rpm (ALS). All serum preparations were conjugated with fluorescein isothiocyanate(6). To serums WS and GP, serum albumin conjugated with Lissamine rhodamine RB 200 was added(7). Serums were stored at 4°C. Preimmunization bleedings were similarly treated.

The cover slips were fixed for 15 minutes in (a) 10% formalin, then washed 3 times with phosphate-buffered saline, pH 7.4, before staining; (b) in acetone, then air-dried; or (c) in methyl alcohol, then air-dried. If the cover slips could not be stained immediately, those fixed in acetone or methyl alcohol were placed at -60°C, and those fixed in formalin at 4°C, either in the formalin or phosphate-buffered saline.

The cover slips were overlaid with the conjugated serums for 30 minutes at 37°C, washed in 3 changes of phosphate-buffered saline, pH 7.2, and mounted in buffered glycerol. A Zeiss fluorescent microscope, with an OSRAM HBO 200 light source and a BG12 exciter and GG4-OG4 barrier filter, was used for all observations.

Results. Conjugated immune and control serums were applied to duplicate sets of infected and noninfected cover slips that had been harvested after 72 hours, when CPE was easily observable, and fixed by one of the 3 technics used. The virus could be demonstrated in all infected preparations. Neither the infected cells treated with control serum nor the noninfected cells treated with immune serum gave specific fluorescence. The viral aggregates were best demonstrated with serums WS and GP to which the counterstain had been added. Formalin-fixed material

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could be stored at 4°C in 10% formalin for periods up to 2 weeks with little or no diminution of fluorescence, while the methanol- and acetone-fixed material had to be examined immediately, as storage even at -60°C did not preserve the antigen. When an inoculum was used that gave maximum virus and CPE at the end of 72 hours but no CPE at 24 and 48 hours, harvested cultures at these times gave specific fluorescence for the viral aggregates; the 24-hour culture showed a marked decrease in number of infected cells, however. With large inocula, virus could be determined as early as 10 hours after infection. Unconjugated immune serum applied to infected cover slips blocked over 90% of the specific fluorescence. Heterologous serums did not produce this effect.

Discussion. An adapted strain of VEE in GPHC can be detected by specific immunofluorescent technic as early as 10 hours when heavily inoculated, and in 24 to 48 hours with an inoculum that does not give a CPE during this period. The whole immune serum (WS) conjugated with fluorescein isothiocyanate with the counterstain added provides the best reagent. Although the methanol-precipitated conjugate (GP) produces similar results, its routine use could present problems, in that extreme care must be taken to prevent denaturation and subsequent loss of specificity. Generally, the stability of viral antigens in formalin has not been studied. It is interesting that our findings show a stability of the antigen fixed in formalin for up to 2 weeks, while with the usual fixatives its stability diminishes rapidly. This is undoubtedly not true of all viral antigens, but it suggests the possibility of studying the pathogenesis of viral diseases in routine pathologic specimens.

Fig. 1 shows the infected cell treated with the immune conjugate. In the infected controls, viral aggregates are demonstrated on the periphery of the cell. Undoubtedly there is other virus present, but it evidently is not in a degree of maturation to be detected. It is believed that there is no problem of penetration of the antibody into the cell, as evidenced by the fact that yellow fever virus can be detected in the perinuclear region(8),

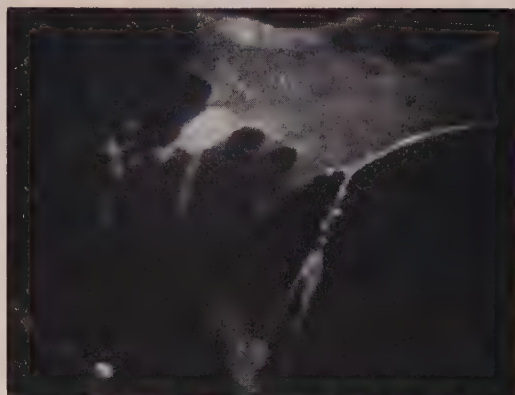


FIG. 1. Infected GPHC treated with conjugated immune whole serum (WS).

and infectious canine hepatitis virus in the intranuclear inclusion bodies(9). The virus cannot be detected prior to 10 hours, possibly because of an eclipse phase. With this virus there may be a breakdown of the virus particles, with a lack of antigenic sites for antibody attachment.

Further studies are in progress to determine the time required for cultures of non-adapted strains of VEE to grow before being demonstrated by this method. If nonadapted strains can be detected with equal facility this procedure could provide a valuable diagnostic tool at the clinical level.

Summary. In a guinea pig heart cell line, infected with an egg-adapted strain of Trinidad VEE, the virus could be demonstrated by immunofluorescence prior to development of CPE. Formalin fixation preserved the antigenicity, and the cover slips could be stored in this fixative at 4°C for periods up to 2 weeks without diminution of specific fluorescence. A whole burro serum conjugated with fluorescein isothiocyanate with a counterstain added proved a more reliable reagent than fractionated or absorbed sera. The model as described has the possibility of obtaining a specific viral diagnosis at the clinical level in the period normally required for initial isolation by conventional cell culture technics.

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Immunologic Studies of Prolactin.* (26290)

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Although the capacity of prolactin to induce antibody formation has been demonstrated(1), no recent studies have been reported in which the antigenicity of relatively small doses of more purified prolactin preparations have been evaluated. A study was therefore designed to evaluate the antigenicity of a relatively pure preparation of prolactin and to investigate the possibility that such antibodies could be employed in a useful assay for this hormone. The present study confirmed the ability of prolactin to induce antibody formation. The antisera that were obtained yielded hemagglutination reactions which were inhibited by small quantities of added prolactin, but which were not inhibited by much larger amounts of other pituitary hormones.

Materials and methods. *Prolactin:* A highly purified preparation of ovine lactogenic hormone with a stated potency of 15 I.U./mg[†] was used. *Immunization.* Adult white rabbits were injected subcutaneously with saline solutions of prolactin suspended in equal volumes of Freund's complete adjuvant. Each of the following amounts of prolactin was given to a different animal: 0.1 mg (Rabbit 1), 2.5 mg (Rabbit 2), 12.5 mg (Rabbit 3). Eleven weeks later boosters of 0.25 mg of prolactin were given to rabbits

2 and 3, and one week subsequent to initial injection, a 0.5 mg booster was given to rabbit 1.[‡] *Sampling:* Peripheral blood samples were obtained from all 3 animals just prior to immunization. Rabbit 1 was bled again 3 weeks later, while multiple samples were obtained from Rabbits 2 and 3 from the third to 12th week after the initial immunization. The antiserum obtained on the 12th week from Rabbit 2 was used in evaluation of the inhibition of hemagglutination. *Hemagglutination titers:* All serum samples were heated to 56°C for 30 minutes to inactivate complement and absorbed with sheep erythrocytes to prevent nonspecific hemagglutination. Antigen-antibody reaction was evaluated by the hemagglutination technic(2). Each 1.0 ml volume of a 2.5% suspension of washed, tannic acid treated sheep erythrocytes was sensitized with 0.2 mg of prolactin. Of this suspension 0.06 ml was added to tubes containing either normal rabbit serum, serial dilutions of antisera, or serial dilutions of test materials in antiserum. Significant hemagglutination was considered to have occurred if at the end of 3 hours the erythrocyte suspension appeared as a granular layer of cells covering the bottom of the tube. Less marked agglutinations were classified as negative.

Results. All animals lacked demonstrable anti-prolactin antibodies prior to immunization; all developed such antibodies after im-

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[†] Armour, Lot Nos. S 10209 and R 10109 supplied through the kindness of Endocrine Study Section, Nat. Inst. of Health, U.S.P.H.S.

[‡] Boosters for Rabbits 2 and 3 were in saline. Booster for Rabbit 1 was in Freund's complete adjuvant.

TABLE I. Inhibition of Prolactin-Anti-Prolactin Hemagglutination.

Substance	Quantity, μ g	Inhibition*
Prolactin†	.026	+
Crude extract, ovine pituitary	.1	+
Somatotropin, bovine	7.8	+
Corticotrophin, commercial	60.0	—
TSH, purified	5.0	—
TSH, Armour, commercial	250.0	—
Human albumin, commercial	"	—
Human immune globulin, commercial	"	—
LH	"	—
FSH	"	—
HGG	"	—

* + values indicate smallest amount found to inhibit the reaction. — values indicate minimum amount of substances which failed to inhibit hemagglutination; larger quantities were not tested.

† Prolactin—original antigen; TSH, purified—highly purified bovine thyrotropin, 5 units/mg, prepared by Dr. John Pierce; LH, FSH, HGG—luteinizing hormone, follicle stimulating hormone and human chorionic gonadotropin, respectively, supplied by the Endocrine Study Section, Nat. Inst. Health, U.S.P.H.S.

munization. Qualitative "ring" tests showed them to be precipitable; quantitative hemagglutination assays showed that Rabbit 3, which was given the largest dose of antigen, had the highest antibody titer in each of the post-immunization bleedings.

The specificity of the hemagglutination caused by the reaction of anti-prolactin antiserum with prolactin-sensitized erythrocytes was evaluated by determining the amount of test substances added to antiserum that inhibited hemagglutination. Significant inhibition was considered to have occurred if there was at least a 4-fold difference between antiserum without inhibitor and an equal volume of antiserum with added inhibitory material. Each experiment was performed at least twice before the findings were assumed to be valid. Table I summarizes the data obtained.

Discussion. The antigenicity of ovine pro-

§ Reported by Endocrine Study Section, Nat. Inst. of Health.

lactin in rabbits confirmed older studies; the ability of as little as 0.1 mg of a purified preparation to induce antibody formation raised the hope that this reaction may have been specific for prolactin. Although *in vitro* cross-reaction was found with small amounts of bovine growth hormone, it should be noted that 300 times more growth hormone than the prolactin was required for inhibition of hemagglutination. Furthermore, bioassay of the growth hormone for prolactin revealed significant contamination.§ A variety of other proteins, including several of the pituitary hormones failed to cross-react. Thus, the present data are consistent with the possibility that the antibodies produced against prolactin were relatively specific.

Recently Simkin and Goodhart, using the pigeon crop bioassay, have reported the presence of prolactin in the blood of human subjects(3). The assay system used in the present study is capable of detecting as little as 0.026 μ g of prolactin. If anti-ovine prolactin antiserum cross-reacts with human prolactin, or if potent and specific antisera can be prepared against human prolactin, then an immuno-assay could be developed that would be sufficiently sensitive to evaluate the prolactin content of human blood.

Summary. Rabbits immunized with relatively pure prolactin developed antibodies against this hormone. The antiserum seemed to be specific for prolactin, and could be used in an assay system capable of detecting as little as 0.026 μ g (0.4 milliunits) of prolactin.

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Immunocytochemical Reaction of Serum of Patients with Hepatic Diseases With Hepatic Structures.* (26291)

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Preceding studies have indicated the presence of gamma globulin and evidence for its local production in livers near areas of destruction of the parenchyma in hepatitis and cirrhosis(1). This stimulated an investigation as to whether the serum of patients with such diseases, and particularly its gamma globulin fraction contain antibodies for structural elements of the liver. The concept that self perpetuation of hepatic cirrhosis results from an immunologic reaction is old but not established(2). Clinical support comes from cases of lupoid hepatitis described as an antigen-antibody disease(3). Serologic evidences in favor of this are 1) the older investigations showing precipitating antibodies against liver tissue in hepatic disease(4,5) and 2) recent studies with the globulin consumption test(6) and demonstration of complement binding antibodies which, however, are not organ specific(7). Immunocytochemically, in 2 of 7 cases of chronic hepatitis, the serum showed binding to the cytoplasm of liver cells(8).

Material and methods. Sera from 32 patients with various hepatic disease, from 10 patients with lupus erythematosus, and from 30 hospital controls (Table I) were studied by the indirect Coon's technic(9) with cryostat sections of 5 human livers, normal human heart, normal human kidney, rabbit and rat liver, kidney and heart. The sections of human livers (1 normal, 1 malignant hepatitis (rapid transition of viral hepatitis to post necrotic cirrhosis), 1 extrahepatic biliary obstruction, 1 primary biliary cirrhosis and 1 active postnecrotic cirrhosis) were fixed in cold acetone and washed with buffered saline at pH 7. A few drops of serum were placed on each tissue section for 30 min. at room temperature. The sections were then

washed with buffered saline for 40 min. Fluoresceinated rabbit antihuman gamma globulin(10) absorbed with liver powder was applied to the sections at room temperature for 30 min. They were washed and mounted in buffered glycerin and examined under the fluorescence microscope. In selected cases gamma globulin was separated from the serum of the patients with a diethylamine-ethylcellulose column(11) and used for comparison with whole serum. In the cases that showed nuclear binding the cryostat sections of liver were incubated with desoxyribonuclease (DNAase) and ribonuclease (RNAase)(12) before application of the serum. The presence of antiblood group substances were determined in all sera. Sera were then absorbed with blood group specific substances A and B and then tested with sections as before.

Results. Binding was encountered to nuclei or to cytoplasm of ductular cells. Nuclear binding (Fig. 1A) was seen with 4 cases of biliary cirrhosis and 2 of malignant hepatitis. It demonstrated all nuclei of all human livers and other human organs as well as of all rat and rabbit organs. It was abolished by pretreatment of the sections with DNAase but not with RNAase. It was present when gamma globulin fractions alone were used instead of serum but was absent when serum minus gamma globulin was applied. In none of the cases was the LE preparation positive. Sera of 2 patients with primary biliary cirrhosis were examined before and after long term corticosteroid therapy. The binding present initially was abolished in one case and diminished in the other. The nuclear binding in lupus erythematosus behaved as that in liver disease except that it did not appear to be influenced by therapy.

Binding to the cytoplasm of ductular cells (Fig. 1B, 1C) was present in 18 cases with various liver diseases and in 1 of 30 hospital controls (Table I). The patient with ob-

* This investigation supported by grant from Nat. Inst. of Arthritis and Metab. Dis., U.S.P.H.S., and The Block Foundation.

structive jaundice and positive binding had a surgical stricture of the common bile duct for a year and a liver biopsy showed extensive fibrosis and ductular cell proliferation. Binding in the ductular cells was either diffuse or granular, the former being more common, and seems to run parallel with the histochemical PAS reaction of these structures after diastase treatment. Occasionally it marked the contents of the ductular lumen and uniformly the basement membrane. Some sera visualized small ducts at times even more than ductules. Cytoplasm of the hepatic parenchymal cell never was bound. Separated gamma globulin demonstrated ductular cells while

TABLE I. Number of Cases and Type of Binding in Various Liver Diseases, in Lupus Erythematosus and in Control Patients.

Diagnosis	No. of cases	No. with nuclear binding	No. with ductular cell binding
Primary biliary cirrhosis	5	4	2*
Malignant hepatitis	3	2	0
Lupus erythematosus	10	10	0
Viral hepatitis	7	0	5
Laennec's cirrhosis	4	0	4
Postnecrotic "	8	0	6
Juvenile "	2	0	2
Extrahepatic biliary obstruction	3	0	1
Hospital controls without hepatic disease	30	0	1

* 1 only after steroid therapy.

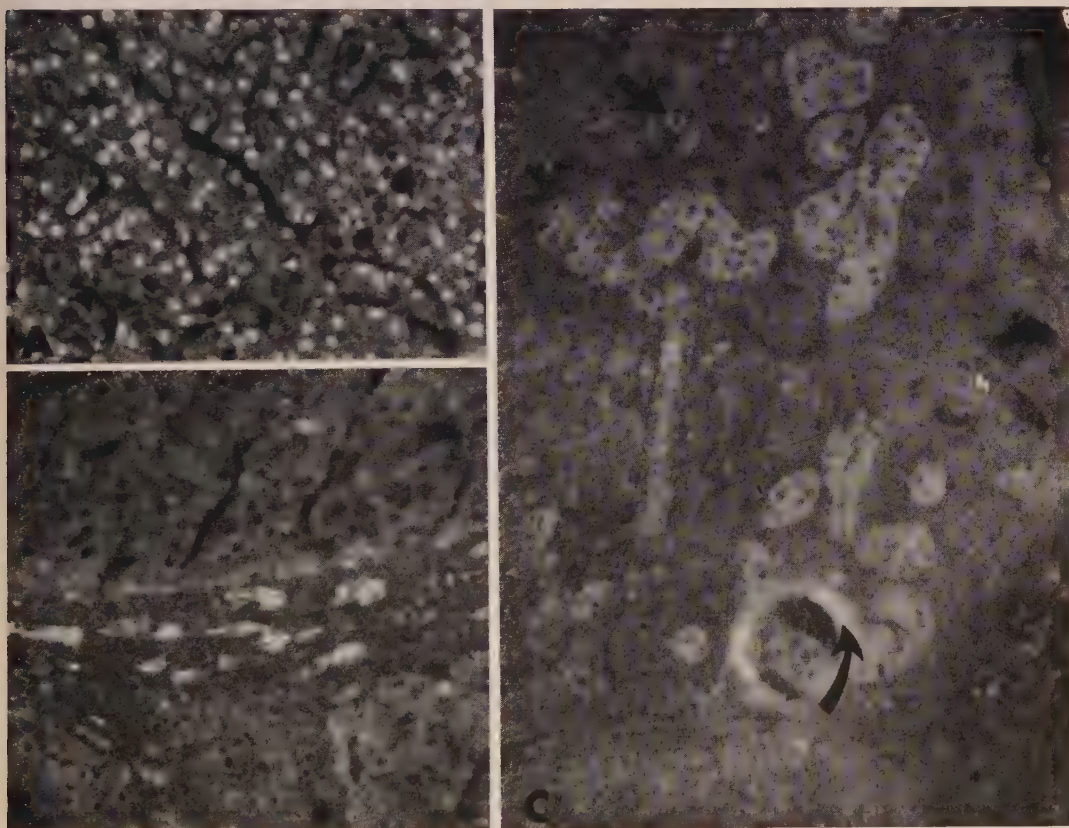


FIG. 1. Fluorescence photomicrographs of cryostat sections of liver after treatment with serum of patients followed by fluoresceinated anti-human gamma globulin antiserum. A. Staining of nuclei of normal human liver by serum of patient with primary biliary cirrhosis ($\times 250$). B. Staining of proliferated ductules of a liver with hepatitis by a serum of a patient with juvenile cirrhosis ($\times 100$). C. Staining of ductules as in (B) by a serum of a patient with chronic hepatitis. Note staining of cytoplasm and not of nuclei and dense staining of bile in the lumen (curved arrow). Straight arrow points to plasmacytoid mesenchymal cells which are also stained by fluoresceinated anti-human gamma globulin without preceding treatment with patient serum ($\times 250$).

serum minus gamma globulin did not. Binding did not correlate with the presence of blood group substances although in some cases it was decreased following absorption of serum with blood group specific substances. Two cases of primary biliary cirrhoses exhibited both nuclear and ductular cell binding, in one simultaneously and in the other after suppression of the nuclear reaction by steroid therapy. Ductular cell binding was most conspicuous with sections of the case of malignant hepatitis and less so in the cases of extrahepatic biliary obstruction and primary biliary cirrhoses. It was not seen in normal liver nor in rat or rabbit liver.

Discussion. Apparently 2 types of reacting substances, presumably antibodies, occur in the serum of patients with liver diseases. One binds nuclei and has neither organ nor species specificity similar to the antinuclear factor found in lupus erythematosus(13,14). However, the cases in question showed no LE phenomena as do cases of lupoid hepatitis, and were either malignant hepatitis or primary biliary cirrhosis. The reaction was abolished or diminished by steroid therapy. Reduction of the reaction by previous treatment with DNAase but not with RNAase suggests that binding occurs with DNA itself or DNA associated proteins as in lupus erythematosus(12). This nuclear binding in liver disease is possibly nonspecific and not pathogenetically related to the disease but may be an anamnestic reaction.

Serum proteins which bind the cytoplasm of the ductules are found in a variety of hepatic diseases with or without nuclear binding. This binding is not influenced by steroid therapy and is directed against material found in a diffuse or sometimes a granular form in the ductular or ductal epithelium and sometimes in the lumen, which means in the bile. Since its appearance parallels granular nonglycogenic PAS positive material in the cytoplasm and in bile, it is either mucopolysaccharide or lipoprotein(15). This is supported by the observation that part of the material seems to be blood group specific substance in nature. Whether it represents an abnormal substance or a retained or aggre-

gated normal substance remains to be established. Even more challenging is the question of a possible pathogenetic role in liver disease of such antigenic material either as a target of an antibody or as an irritating antigen-antibody complex.

Summary. Binding of sera of 32 patients with various liver diseases, 10 patients with lupus erythematosus and 30 hospital controls to human and animal livers and other organs was studied using the indirect Coons' fluorescent antibody technic. Two types of circulating reacting substances were demonstrated: one noted in patients with primary biliary cirrhosis and malignant hepatitis bound nuclei without species or organ specificity; the other occurring in a variety of hepatic diseases reacted with the cytoplasm of ductule cells. The former was influenced by steroid therapy while the latter was not. The antigenic material in the ductular cell possibly is a muco- or lipoprotein which may also be present in bile.

The authors are indebted to Mrs. Esther Trachtenberg for enthusiastic technical support.

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Evidence of Prenatal Heterosis Relating to X-Ray Induced Congenital Anomalies.* (26292)

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Heterosis or hybrid vigor has been established for adult mice with respect to their tolerance of ionizing radiations (Rugh and Wolff(1). It has also been studied with respect to single organ (testis) reaction where it was found that the resistance of the gonad in the hybrid was more like a simple dominant relation than synergistic heterosis (Rugh, Funk and Wohlfromm(2). Since heterosis can be demonstrated at sexual maturity (2 months of age in the mouse) it seemed pertinent to determine whether it existed prior to birth, and at a time when ionizing radiations quite consistently caused resorptions and congenital anomalies. Thus, this study was designed to establish the resistance of the hybrid to irradiation effects caused by 200 r x-rays at the period of greatest neurogenesis, namely 8.5 days gestation.

Materials and method. The animals used were the CFI Swiss white stock from Carworth Farms, the C57 black/6 stock from Jackson Memorial Laboratory, Bar Harbor, Me., and their F₁ hybrids resulting from the cross between the CFI females and C57 males. This cross is easily made and results in highly viable offspring.

Matings were begun at 5 p.m. The males were removed at 9 a.m. and each exposed female was examined for presence of a vaginal plug. Those with such indications of mating were segregated and at 8.5 days the experimental animals were subjected to 200 r x-rays. The controls, of the same stage of pregnancy, were kept under identical condi-

tions but were not irradiated. At 18.5 days gestation age each of the experimental and control females was killed by cervical dislocation, the uteri were immediately excised, and each fetus, or implantation site, was dissected out and examined. In this way, every successfully fertilized egg which reached the uterus and established an implantation site (at 4.5 days) could be included in the total counts. Each of some 2630 implantation sites in 302 pregnancies was counted as to whether it was normal, resorbed, dead, or showed anomalous development. The anomalies included stunting, exencephaly, anencephaly, protruding intestine, and prominent eye defects. The "normal" mice were so designated when, from all evidence, they appeared normal as to size, development, and absence of obvious anomaly. Mice at 18.5 days are essentially at the delivery stage of development (normally 20-21 days) but if allowed to reach delivery, the mother would generally kill and eat those which are abnormal. Thus, by studying the fetuses *in utero* we are able to present data on total irradiation effect.

The irradiation facilities used included a single x-ray tube at 48.5 cm distance to the position of the gravid uterus, the radiation interrupted by 0.28 mm Cu. and 0.50 mm Al. filters so that the output in air at the level of the 8.5 day embryos was 50 r/minute. Since the mouse is relatively small and the embryos are close to the body surface, extrapolation to rads is well within the maximum range of 3%. The total dose was 200 r in 4 minutes.

Experimental data. Table I presents an adequate number of pregnancies and implantations for statistical significance. Although

* Based on work performed under contract for U. S. Atomic Energy Commission and aided by U.S.P.H.S. Grant #A-3045.

TABLE I.

	Cross (mice)	Preg- nancies	Implanta- tions	Normal	Resorptions	Dead	Anomalies
Controls—no irradiation	CF1 × CF1	61	630	591 (94)	37 (5.7)	2 (0.3)	0
	CF1 × C57	39	348	325 (93)	31 (9)	1 (0.5)	1 (0.5)
	C57 × C57	33	253	203 (80)	31 (12)	9 (3.5)	10 (4)
X-rays: 200 r @ 8.5 days	CF1 × CF1	74	704	270 (38)	274 (39)	33 (4)	127 (18)
	CF1 × C57	58	315	137 (44)	96 (30)	22 (7)	51 (17)
	C57 × C57	37	380	18 (5)	334 (90)	20 (5)	10 (2)

Percentage of total implantations given in parentheses.

the C57 stock does not mate readily and the general fertility is low, among the successful matings there were 253 control and 380 x-rayed implantations available for this analysis.

Among the controls (with no irradiation) one would expect a higher rate of resorptions and anomalies among the C57 stock than among the CF1 stock, but note that among the hybrids there was an intermediate level of resorptions (9%) between controls (5.7%) and pure C57 stock (14%). Anomalies appeared much more frequently among the C57 mice. These included eye anomalies which apparently occur in 8-10% of all mice from this stock (Dickie(3)). Thus, among the normal (control) mice of pure or hybrid crosses the C57 strain showed a higher level of intra-uterine resorptions and anomalies than did either the hybrids (which included the C57 genes) or the pure CF1 stock.

The incidence of anomalies in the CF1 strain has been reported elsewhere (Rugh and Grupp(4,5,6)), but to the figures are added those from more recent observations. These do not alter the percentage values, but increase the totals involved. The normal fetuses among the hybrids are higher by 6% (44% over 38%) than among the pure CF1, and far higher than among the pure C57 (5%), thus a slight indication of heterosis. This is further substantiated by the lower incidence of resorptions among the hybrids, 30% as contrasted with 39% for the CF1 and 90% for the C57 stock. The incidence of anomalies among the hybrids and the CF1 strains is about the same, and somewhat higher than among the C57 mice simply because most of these implantations resulted in

resorptions (90%). Thus, there appears to be clear cut evidence that resistance to 200 r x-rays at 8.5 days gestation is somewhat better in the hybrids than in either of the parental stocks, both with respect to percentage normals and percentage resorptions. Heterosis, therefore, extends back to the 8.5 day stage, at least with respect to the damaging effect of ionizing x-rays.

Discussion. Heterosis is generally measured by overall survival, longevity, productivity, etc. whether or not altered by some traumatic experience, but always compared with the parental stocks. There is no evidence that this hybrid vigor extends to further generations, but it seems to be a rather general rule for all extensive heterozygotic conditions. The heterozygote is therefore not a blending of certain potentialities, but may be actually a synergistic effect resulting in a more viable generation. If one crosses these 2 strains and tests their adult hybrid offspring against the same age pure strains, the hybrids show a much greater resistance to the lethal effects of ionizing radiations than does either parental strain. There is a considerable difference between the 2 pure strains, but neither is as radioresistant as their hybrid offspring. This resistance must be due to composite effects, certainly more than one because irradiation death is never simple. Irradiation death may be due to hemorrhage, to epithelial destruction, to edema and excretory malfunction, to secondary infection, to extensive and prolonged anemia, etc. But some of these causes must be ruled out in the embryo or fetus because they could not (and do not) exist. Nevertheless, there appears to be evidence herein presented that the hybrid between the CF1 and

the C57 strains, even at 8.5 days gestation, seems to withstand the trauma of ionizing radiations better than either of the parental strains, as measured by subsequent embryonic development.

This study points up one more factor that may be overlooked, namely that heterosis shifts percentages toward a higher survival, but this includes a higher level of anomalies than among the very sensitive C57 strain. Thus, one sees here an example of heterozygosity aiding the survival of anomalies.

One might raise the question of the effect of reversing the hybrid cross, mating the CFl males with C57 females. Genetically there should be no difference, but it must be admitted that any cytoplasmic effects on the very early development might be involved. However, since the exposure to x-rays occurred long after implantation (which occurs at 4.5 days when the germ layers are forming) it can be assumed that any differences relating to the origin of the cytoplasm would have disappeared. Possibly heterotic influences are present at the moment of fertilization, only to be manifest later when tests are possible. Certainly they appear to be working at 8.5 days so that the more complicated investigations of earlier stages seem indicated.

Summary and conclusions. 1. Embryonic mice at 8.5 days gestation from pure C57 and CFl strains, and their hybrids, were x-irradiated to 200 r to determine whether prenatal

heterosis was manifest. Criteria were the number of normal, resorbed, dead, or anomalous mice seen at 18.5 days in the hybrids as compared with the pure lines. 2. The hybrids at 8.5 days exhibited some degree of heterosis based upon the higher percentage of normal fetuses, and the lower percentage of resorptions when comparisons are made with the pure strain parental stocks. 3. For heterosis to be manifest, the hybrids had to overcome the severely deleterious effect of the presence of C57 genes as indicated by the 90% resorptions among this parental stock and only 30% in the hybrids, while the CFl showed 39%. Thus, there is evidence of more than mere dominance of the more resistant strain genes, hence heterosis at least by the time of organogenesis. 4. The heterosis for the embryo results in better survival, hence better survival also of the x-irradiation-produced congenital anomalies.

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Excretion of Urinary N-C¹⁴-Methyl Morphine and Pulmonary C¹⁴O₂ in the Monkey and Dog after Subcutaneous Injection of the Labelled Drug.* (26293)

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The application of radioisotopic tracer technics has provided much useful information on the biological disposition of the potent analgesic agents. March and Elliot(5), Elliot *et al.*(4), and Achor and Geiling(1) have studied the physiological disposition of C¹⁴-labelled morphine in man, in rats and

in mice, respectively. The present report of-

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TABLE I. Urinary Excretion of Free and Bound Morphine and Exhaled C¹⁴O₂ in Monkey and Dog for the First 24 Hours after Subcutaneous Injection of 2.0 mg/kg of N-C¹⁴-methyl Morphine.

	% of administered drug			
	Monkey		Dog	
	378	384	1	2
Urinary excretion*				
Free	6	14	13	15
Bound	55	71	48	52
Total	61	85	61	67
C ¹⁴ O ₂ excretion	1.5	.9	.2	.2

* Urine was cage collected as described in text. Dogs and monkeys were catheterized at the end of 24 hr period and urine so obtained was combined with the cage collected sample.

fers details of studies on physiological disposition of N-C¹⁴-methyl morphine in monkeys and dogs.

Methods and materials. The N-C¹⁴-methyl labelled morphine employed was synthesized according to the method of Andersen and Woods(2) and had a specific activity of about 2 μ C/mg.

The studies on urinary excretion and C¹⁴O₂ elimination were done according to the method of Woods *et al.*(9). Estimation of morphine in urine was performed according to the method of Mulé and Woods(7). **Animal studies:** Female animals were used throughout the study. Monkeys or dogs received 2.0 mg/kg of N-C¹⁴-methyl morphine,[‡] subcutaneously, and were placed immediately in the CO₂ collection apparatus. Both urine and C¹⁴O₂ were collected for a 24-hour period. The animals were catheterized at the end of this interval and urine so obtained was added to the pan collected material.

Results. Urinary C¹⁴-morphine and pulmonary C¹⁴O₂ in dogs and monkeys. The results on urinary excretion of N-C¹⁴-methyl morphine and elimination of C¹⁴O₂ in the monkey and dog are summarized in Table I. Urinary excretion of free morphine 6-14%, bound morphine 55-71% and total morphine 61-85% in the monkey agrees well with the results of Mellett and Woods(6) on urinary excretion of morphine in non-tolerant mon-

keys given 15 mg/kg of morphine twice daily (free 10 ± 4.9 , bound 67 ± 24.6 and total 77 ± 16.7). It would appear from these data that in the range of 2.0 to 30.0 mg/kg of morphine, total dose given does not significantly alter the pattern of drug metabolism in the monkey.

The results of C¹⁴O₂ elimination in the monkey are of interest because the values appear to be very low when compared with C¹⁴O₂ elimination after administration of N-C¹⁴-methyl levorphanol, (0.9-1.5% for morphine *vs.* 17 to 24% for levorphanol) (Woods *et al.*, (9)).§

Urinary excretion of free morphine (13-15%), bound morphine (48-52%) and total morphine (61-67%) agree well with the figures of Cochin, *et al.*(3) free $14 \pm 3\%$, bound 56 ± 9 , and total $70 \pm 1.1\%$. In the study of Cochin *et al.* non-tolerant dogs were employed and received a dose of 30 mg/kg, subcutaneously. These data would again indicate that the magnitude of the total dose in a range of 2.0 to 30 mg/kg does not significantly alter the drug metabolism pattern in this species.

C¹⁴O₂ elimination by the dog after N-C¹⁴-methyl morphine (0.2%) is similar to the low excretion of C¹⁴O₂ found in this species after N-C¹⁴-methyl levorphanol (1.0-1.5%).

Discussion. A number of factors may influence the pattern of metabolism of a given drug or series of drugs in various species. One of the factors generally considered important in modification of metabolism pattern is the dose. The results of these studies and those studies of Mellett and Woods(6) and Cochin *et al.*(3) indicate that in a dose range of 2.0 to 30 mg/kg of morphine there is no significant alteration in metabolic patterns of dogs or monkeys.

There is, of course, a wide variation in the manner in which various species metabolize morphine. However, within a given species closely related congeners of morphine may also be metabolized in quite different fashions. For example the monkey is capable of

[‡] All doses are calculated as the free base although the drug was administered as a solution of the sulfate salt.

§ The same 2 animals were employed in both levorphanol and morphine studies after an interval of one year.

extensive N-demethylation of levorphanol (20%) as estimated by $C^{14}O_2$ elimination as compared with a limited ability to N-demethylate morphine (ca. 1%). The dog on the other hand appears to have little ability to N-demethylate either levorphanol or morphine. It is interesting that a similar pattern of O-demethylation of codeine obtains in the dog and monkey, *i.e.*, the monkey has a moderate capacity (8.0% of the dose) for O-demethylation while the dog has little or no capacity for this function (Woods *et al.* (8)).

It is apparent, therefore, that small changes in chemical configuration are capable of extensive modification of the metabolic pattern of the potent analgesics.

Summary. The results of urinary excretion and $C^{14}O_2$ elimination experiments in dogs and monkeys receiving 2.0 mg/kg of N- C^{14} -methyl morphine, subcutaneously, were as follows: dogs excreted 13-15% of total dose as free morphine and 48-52% as conjugated morphine and about 0.2% as exhaled $C^{14}O_2$. Monkeys excreted 6-14% as

free morphine and 55-71% as conjugated morphine and about 1% as exhaled $C^{14}O_2$.

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Growth of Certain Arthropod-Borne Viruses in Hamster Kidney Tissue Culture. (26294)

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In recent years investigators have shown successful propagation of selected arthropod-borne viruses in various tissue culture systems(1-5) including hamster kidney tissue culture (HKTC). A single tissue culture system sensitive to many of the arthropod-borne viruses is particularly desirable. In our laboratory a long term study is being made to evaluate tissue cultures as host systems for isolation of viruses, study of virus cell relationships, and production of viral reagents. It has been our experience that hamster kidney (HK) cells are outstanding among the many cell lines under investigation in serving as a culture medium for an increasing number of viruses.

To evaluate HKTC as a suitable standard cell system for arthropod-borne viruses in respect to cytopathogenic effect (CPE), sensitivity, and viral growth, the present studies were made with 4 group A viruses: eastern equine encephalomyelitis (EEE), western equine encephalomyelitis (WEE), Sindbis, and Mayaro; and 5 group B viruses: Ilheus, St. Louis encephalitis (SLE), Japanese encephalitis (JE), Murray Valley encephalitis (MVE), and West Nile (WN).

Methods. Tissue cultures (TC) were prepared essentially according to Youngner's (6) technic. After 5-15 days, cultures containing full cell sheets and 1.5 ml of maintenance medium(7), were inoculated with 0.3

ml of a 10^{-3} dilution of virus material. Passes were made from inoculated cultures by transferring 0.3 ml of the fluid phase to fresh monolayer HKTC. Passes in TC were made according to viral activity indicated by CPE, and the last 2 passes were made during the period of greatest viral concentration previously determined by growth curves. In a few instances the fluid phase containing virus was frozen and stored at -70°C until inoculated into mice or TC. No material was stored longer than 10 days.

Comparative titrations were made using the same serial 10-fold dilutions and inoculating 0.03 ml intracerebrally into each of 5 mice per dilution and 0.3 ml into each of 5 TC tubes per dilution. Tissue cultures were observed 7 days, and those showing CPE were recorded as positive. Mice which died within a 2-14 day observation period were recorded as infected with virus. Samples taken for growth and inactivation curves were considered undiluted material from which 10-fold dilutions were made. No adjustment in titer was made to compensate for the greater inoculum in TC.

The viruses used for the initial passage in TC and for growth and inactivation curves were from infected mouse brain suspensions which had been stored in sealed glass ampoules in a dry ice box. Six of the viruses (EEE, WEE, WN, SLE, JE, and MVE) used in this study were obtained from the American Type Culture Collection, Washington, D. C., and are described in the Viral and Rickettsial Registry(8). Sindbis(9) and Mayaro(10) viruses were obtained from Dr. Theiler of Rockefeller Inst. for Medical Research. Ilheus(11) virus was supplied by Dr. McCrumb of Univ. of Maryland.

Rate of viral growth was estimated by titration of the fluid phase from inoculated HKTC after various periods of incubation. Titrations of group A viruses were made in TC; other viruses were titrated in mice. Cultures having a full monolayer cell sheet were inoculated with 0.3 ml of a 10^{-2} dilution of virus. A sample of the fluid from 2 tubes was then immediately pooled and frozen until titrated. Inoculated tubes were

incubated at 35°C for 30-45 minutes to allow time for possible adsorption of virus. The excess inoculum was then removed and cultures were washed 4 times with 1 ml volumes of TC maintenance medium to remove unadsorbed virus. The last wash was saved for titration and on charts represents the 0 hr sample. The cultures were then replenished with 1.5 ml of maintenance medium, and incubation at 35°C continued. At 4 or 12 hour intervals during a 7-day period 2 tubes were selected at random and the supernatant fluid pooled and titrated.

Data for plotting inactivation curves were obtained by titration of virus at intervals during incubation at 35°C in TC maintenance medium without cells. Tubes containing 1.5 ml of medium were inoculated with virus and the fluid from 2 of these tubes pooled, frozen, and stored at -70°C . Subsequent samples were taken by pooling and freezing the fluid from 2 additional tubes at each sampling time during a 10-day period. Titrations of the stored frozen samples were made in 10-12 g mice.

After 10-12 passages of virus in TC, dilutions of each virus were tested for neutralization by a constant concentration of specific homologous antiserum previously shown to have a neutralization index of 2.0 or greater in mice. Antisera were prepared by repeatedly inoculating rabbits with mouse brain suspensions of virus which had not previously been passed in TC.

Results. The viruses used in this study each caused a CPE which was reproducible through 10-15 passages in HKTC with only slight variation in intensity and time required to cause observable changes in cells. The most variable results were shown by MVE virus which caused less CPE than the other viruses studied. It was found that these viruses could be grouped into 3 categories according to time required to cause CPE. The group A viruses: EEE, WEE, Sindbis, and Mayaro produced the most marked CPE, causing complete destruction of cells in 48 hours or less. The second category included the group B viruses: SLE, JE, MVE, and WN and gave marked CPE,

TABLE I. Log LD₅₀ Titer in Mice and Tissue Culture of Arthropod-Borne Viruses after Passage in Mouse Brain.

Virus	No. MB passages	Titer in mice (per .03 ml)	Titer in HKTC (per 0.3 ml)
EEE	460	8.2	9.4
WEE	25	8.3	8.4
Sindbis	5	5.8	5.3
Mayaro	10	6.6	7.0
Ilheus	28-35	8.5	8.0
SLE	107	7.8	8.0
JE	45	8.7	7.5
MVE	12	7.6	8.5
WN	30	7.4	6.6

usually progressing to 75% cell degeneration after 3-5 days incubation. The CPE caused by Ilheus virus was different in that it usually involved 75% of the cells within 48-56 hours but did not consistently affect the entire cell sheet. It was found that the CPE produced by JE virus was increased in intensity by rapid passages of unfrozen virus laden TC fluid in fresh HK cells so that complete cell degeneration occurred within 24-36 hours after inoculation. This may have been caused by increase of virus in the inoculum although it did not occur with the other viruses.

The titer in TC of mouse brain virus material did not differ widely from its titer in mice (Table I). Control TC inoculated with a suspension of normal mouse brain did not show cell degeneration.

After 10 or more passages in TC marked loss of pathogenicity for mice was noted for each of the viruses as evidenced by much higher titers in TC than in mice (Table II). Exceptionally low titers in mice, as com-

TABLE II. Log LD₅₀ Titer in Mice and Tissue Culture of Arthropod-Borne Viruses after Passage in Hamster Kidney Tissue Cultures.

Virus	No. MB passages	No. TC passages	Titer in mice (per .03 ml)	Titer in HKTC (per .3 ml)
EEE	460	15	5.2	7.5
WEE	25	11	4.7	7.2
Sindbis	5	11	6.0	7.5
Mayaro	10	11	5.8	6.6
Ilheus	28-35	12	2.8	7.3
SLE	107	12	2.5	7.0
JE	45	12	4.8	7.3
MVE	12	12	2.3	7.4
WN	30	12	4.8	7.8

pared to titers in TC, were obtained with Ilheus, SLE, and MVE viruses.

Growth of group A viruses was very rapid. Eastern equine encephalomyelitis virus attained the greatest concentration in the fluid phase of TC 16 hours after inoculation when it had increased in titer from 2.6 log TCID₅₀ at 0 hours to 9.4 log TCID₅₀, while peak growth of WEE, Sindbis, and Mayaro viruses occurred within 24-48 hours (Fig. 1). Group A viruses increased considerably more in titer than Group B's with the exception of Ilheus virus which increased in titer from 1.0 to 7.7 log TCID₅₀ with 75% CPE in 56 hours (Fig. 1, 2, 3). The CPE caused by the group A viruses had affected about 50% of the cell sheet at time of greatest viral concentration whereas the CPE caused by group B viruses (SLE, JE, MVE, and WN) involved 25% of the cell sheet when highest titers occurred.

Titration of samples of virus taken during inactivation resulted in the rates of inactivation shown in Fig. 1, 2, and 3. It was found that the viruses were essentially inactivated after 5 days at 35°C in TC maintenance medium. It is possible that the concentration of virus in the initial suspension may have had some effect on rate of drop in titer. The group B viruses did not persist so long in cell free media, being completely inactivated after 3 days while with group A viruses trace amounts of virus could still be detected at 5 days.

Neutralization tests of the TC passage material in TC resulted in neutralization of 2-5 logs of virus with the exception of SLE virus. The SLE tissue culture material was not neutralized in mice or TC unless it was first passed twice in mice. It was then neutralized in mice but no CPE was prevented in TC.

Discussion. The results presented here indicate that HK cells are particularly suitable as a culture medium for many arthropod-borne viruses. Since the viruses used in this study were all mouse adapted strains, the results are not necessarily applicable to viruses isolated from naturally infected hosts. However, these results provide an indication of probable usefulness of HKTC in isolation

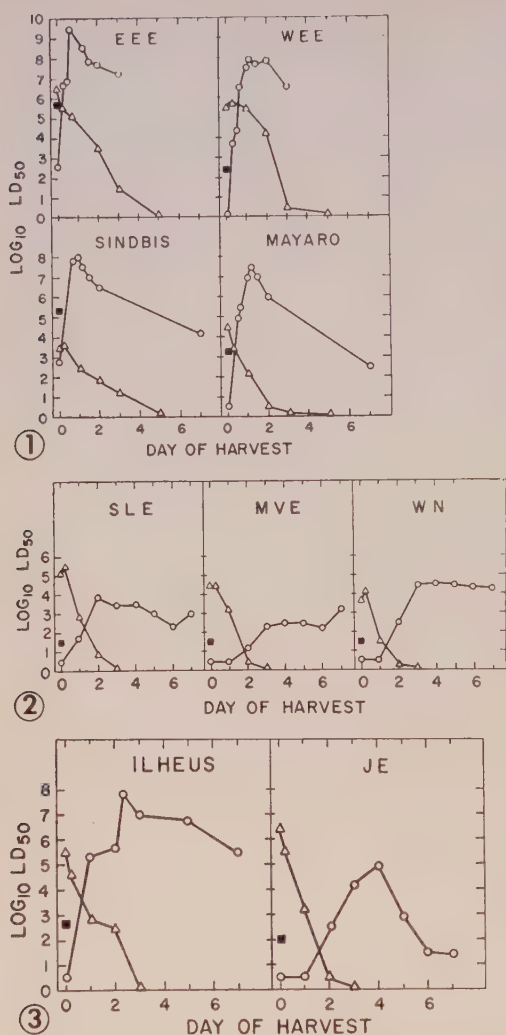


FIG. 1. Growth in hamster kidney tissue culture and inactivation in tissue culture medium without cells of eastern equine encephalomyelitis, western equine encephalomyelitis, Sindbis, and Mayaro viruses.

FIG. 2. Growth in hamster kidney tissue culture and inactivation in tissue culture medium without cells of St. Louis encephalitis, Murray Valley encephalitis, and West Nile viruses.

FIG. 3. Growth in hamster kidney tissue culture and inactivation in tissue culture medium without cells of Ilheus and Japanese encephalitis viruses.

○ = growth; △ = inactivation; ■ = amount of virus in growth curve cultures before washing to remove unadsorbed virus.

work. Isolation of a type 2 dengue virus in HKTC has been reported (12), and Schmidt *et al.* (13) found HK cells to be the most sensitive of 7 cell lines tested when working with newly isolated Uruma virus. In the

present work HK cells were equally sensitive to either mouse adapted or TC adapted strains of virus whereas mice were more sensitive to mouse adapted virus so that HKTC might detect variants of arthropod-borne viruses which would be avirulent in mice.

After the work presented had been completed, a contaminating organism was isolated from apparently normal HK cell lots. This contaminant was suppressed by antibiotics in the TC medium so that it was undetectable until increased by passage, causing a virus-like CPE in HK cells. It could not be grown in bacteriological media until passed 2 or 3 times in TC without antibiotics. The contaminant is tentatively referred to as "hamster agent" and appears to be a bacterial L form.

It occurred to us that the TC passages used in this work may also have contained the "hamster agent." When the last TC passage of each of the viruses was checked, it was found that all except EEE were contaminated with this organism. However this "hamster agent" although present was not responsible for the CPE in titrations of the various viruses since all but SLE were neutralized in TC by antisera which had been prepared against mouse brain viral antigen. In the case of SLE it was shown that the TC titer end-point was due to this "hamster agent" thus explaining the lack of neutralization with anti-SLE serum when tested in TC.

That the presence of the "hamster agent" did not influence virus growth was shown by equal virus titers when the virus was propagated in agent contaminated and uncontaminated cultures.

The significance of the contaminant is not presently known, but its previously undetected presence, the virus-like CPE, and the fact that it may be present in hamsters may be of considerable importance whenever hamster cell cultures are used in virus work.

Summary. An investigation of certain group A and B arthropod-borne viruses in HKTC indicated that in general the group A viruses gave greater CPE and increased in titer more rapidly than the group B viruses studied. Comparative titrations in mice and TC were approximately equal for mouse

adapted strains of virus, but after passage in TC, all the viruses showed some loss of pathogenicity for mice.

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A Biochemical Comparison of 6-Aminopenicillanic Acid, Benzylpenicillin, and 2,6-Dimethoxyphenylpenicillin. (26295)

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With the advent of many new semi-synthetic penicillins as the result of the availability of 6-aminopenicillanic acid(1) as precursor material, it will be possible to examine in greater detail the problem of correlation of chemical structure of the penicillins with their biological and biochemical activities. In this paper one of the newer penicillin derivatives, 2,6-dimethoxyphenylpenicillin, which has shown promise in treatment of penicillin-resistant staphylococcal infections(2,3), benzylpenicillin, and 6-aminopenicillanic acid are compared as substrates for penicillinase, as growth-inhibitory agents, as inducers of penicillinase, and as selectors of penicillin-resistant mutants.

Materials and methods. 2,6-Dimethoxyphenylpenicillin (DMP)* was supplied by Bristol Laboratories, benzylpenicillin (penicillin G) by Squibb Research Inst., and 6-aminopenicillanic acid (6-APA) by Pfizer Research Laboratories. *Hydrolysis* of penicillin by penicillinase was measured manometrically in the Warburg apparatus through

liberation of CO₂ from a NaHCO₃ buffer system by formation of penicilloic acid(4). The enzymes used were cell-free preparations of penicillinase obtained from *Bacillus cereus* and from *Staphylococcus aureus*, which were partially purified and concentrated by isoelectric precipitation(5). *Minimum* growth-inhibitory concentration of penicillin was determined by turbidimetric observations, over a 5-hour period, of the rate of growth of cultures in trypticase soy broth(6). The cultures were shaken in an air-bath (New Brunswick Gyrotory) at 37°C in 300 ml Erlenmeyer flasks equipped with side-attached test tubes for optical density measurements (Bellco Glass, Inc.). The test organisms were penicillinase-producing strains of *B. cereus* (ATCC 10876; NRRL B-569) and *S. aureus*(7). Induced formation of penicillinase by *B. cereus* and by *S. aureus* was carried out as described previously(7). *Selection* of strains resistant to penicillin was accomplished by serial passage of the bacteria through media containing increasingly higher concentrations of the antibiotic(8). Both penicillin-sensitive and penicillin-resis-

* 6-(2,6-dimethoxybenzamido)penicillanic acid (Staphicillin, Celbenin).

TABLE I. Relative Rates of Hydrolysis of Several Penicillins by 2 Different Penicillinases.

Compound	Source of penicillinase	
	<i>B. cereus</i>	<i>S. aureus</i>
Penicillin G	100	100
DMP	1.6	.35
6-APA	.8	.1

Rates of enzymatic hydrolysis of the various penicillins, at 0.01 M concentrations, were measured by rate of release of CO₂ from a NaHCO₃/CO₂ buffer system (pH 7.4) at 37°C in presence of 10⁻³M p-chloromercuribenzoate and compared relative to the rate for penicillin G. Ratios were calculated from the initial linear portions of the reaction curves. The penicillinase solutions were cell-free preparations, assaying 10 to 100 enzyme units (μ moles penicillin G hydrolyzed/hr) per ml. The extracellular enzyme obtained directly from *B. cereus* was concentrated by isoelectric precipitation. The intracellular enzyme from *S. aureus* was released into solution by means of the Nossal bacterial cell disintegrator and concentrated also by isoelectric precipitation.

6-APA = 6-aminopenicillanic acid; DMP = 2, 6-dimethoxyphenylpenicillin.

tant strains of *S. aureus* were employed.

Results. *Relative susceptibility of penicillins to destruction by penicillinase.* The intracellular penicillinase of *S. aureus* and the extracellular penicillinase of *B. cereus*, although different in some of their physicochemical properties(5), attack penicillin in the same manner. DMP was hydrolyzed by staphylococcal penicillinase at a very low rate compared to the rate for benzylpenicillin, the ratio being about 1 to 300 (Table I). The rate of hydrolysis of 6-APA was even less, the ratio to benzylpenicillin being about 1 to 1000. Batchelor *et al.*(1) had found 6-APA to be destroyed by the penicillinase from *B. cereus* similarly at a much slower rate than benzylpenicillin. On comparing the 2 enzymes, it was observed that rates of hydrolysis of DMP and of 6-APA by the penicillinase from *B. cereus*, although still quite low, were 4 to 8-fold greater, relative to penicillin G, than by the enzyme from *S. aureus*.

Inhibition of growth of B. cereus and S. aureus by penicillins. Inhibitory levels of the penicillins against *B. cereus* and *S. aureus* were determined by their effect on rate of growth of the organism. The minimal concentrations of the 3 compounds required for inhibition of *S. aureus* covered a wide

range (Table II). For the large number of organisms used in the experiment, DMP was effective at a concentration less than 1 to 1000 that of penicillin G. Surprisingly, 6-APA was significantly more effective than penicillin G. Against *B. cereus*, however, DMP and benzyl-penicillin were about equally effective, whereas 6-APA was rather ineffective.

Induction of penicillinase in B. cereus and S. aureus by penicillins. Penicillinase-producing cultures of *S. aureus*, as well as *B. cereus*, are stimulated by the presence of penicillin G to elaborate penicillinase many times the basal level(7). In Fig. 1 is shown the rate of enzyme formation in *S. aureus* resulting from various inducing concentrations of penicillin G. It can be seen that the rate of production of enzyme was dependent on the initial concentration of penicillin. From Fig. 2 it is apparent that there was a similar pattern when the formation of penicillinase in *S. aureus* was induced by DMP.

The concentration required to obtain half the maximum rate of enzyme formation which can be achieved is considered as the "induction constant"(9). In Table III are summarized the "induction constants" for the 3 penicillins, with both *B. cereus* and *S. aureus* obtained from graphs in which relative amounts of enzyme formed were plotted against inducing concentrations of the penicillin (cf. 7). For *B. cereus*, benzylpenicillin

TABLE II. Growth-Inhibitory Concentrations of Several Penicillins for Two Organisms.

Antibiotic	Organism	
	<i>B. cereus</i> , μ g/ml	<i>S. aureus</i> , μ g/ml
Penicillin G	20	4,000
DMP	20	2
6-APA	2,000	200

Bacteriostatic concentrations of the 3 penicillins were determined by turbidimetric measurements of rate of growth at 37°C of shaking cultures of *B. cereus* and *S. aureus*. The test systems containing a series of penicillin concentrations were seeded with 1-100 dilution of over-night culture to yield an initial concentration of about 10⁷ organisms/ml. Under these conditions growth was exponential. The generation time for *B. cereus* was about 30 min. and for *S. aureus* about 50 min. The bacteriostatic level is defined as the minimal concentration of antibiotic in which there was no significant increase in turbidity of the culture in 5 hr.

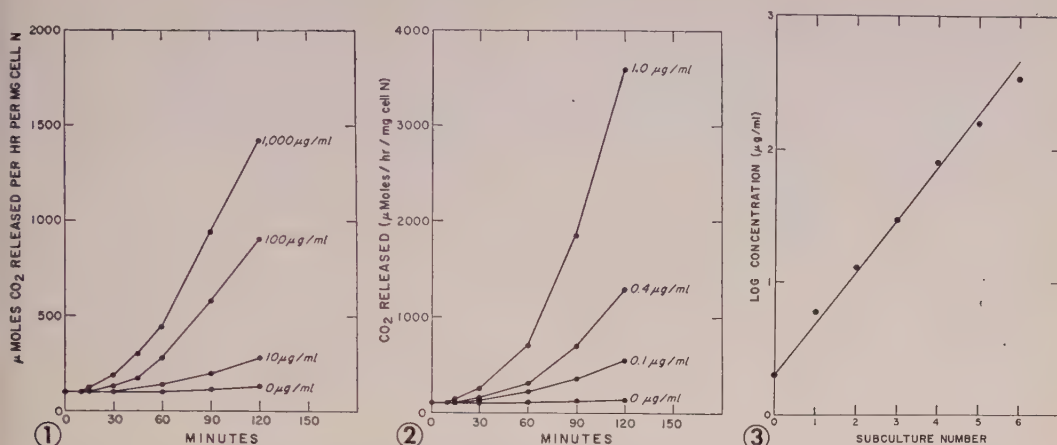


FIG. 1. Induced formation of penicillinase by *S. aureus* in response to various concentrations of benzylpenicillin. Test organisms were washed cells from an overnight culture in trypticase soy broth and were added at an initial concentration of 50 μg (dry wt)/ml. Induction was carried out in trypticase soy broth containing from 10 μg to 1,000 μg penicillin G/ml in an incubated shaker at 37°C. Samples were removed from a 20 ml reaction mixture at periodic intervals and the induction reaction was halted by inactivation of the organisms with 10⁻³M p-chloromercuribenzoate. The penicillinase content thus stabilized was determined manometrically. The resultant penicillinase activities, corrected for growth during the course of the reaction, were plotted against time of withdrawal of the sample.

FIG. 2. Formation of induced penicillinase by *S. aureus* in response to various concentrations of 2,6-dimethoxyphenylpenicillin. The procedure was that described in the legend of Fig. 1. Data are shown for inducing concentrations ranging from 0.1 μg to 1 μg/ml. Above 1 μg/ml the staphylocidal action of the drug inhibited the induction reaction.

FIG. 3. Development of resistance to 2,6-dimethoxyphenylpenicillin by *S. aureus*. An overnight culture of a penicillinase-producing strain of *S. aureus* was inoculated, at a 1-100 dilution, into fresh trypticase soy broth medium. After a 2 hr incubation period, in which the concentration of organisms increased to about 6×10^7 cells/ml, the staphylococci were inoculated, at a 1-10 dilution, into tubes of trypticase soy broth containing a series of concentrations of 2,6-dimethoxyphenylpenicillin ranging from 1 μg to 10 μg/ml. Cultures were incubated at 37°C for 24 hr without shaking. Subcultures, at a 1-100 dilution, were made of the tubes containing the highest concentrations of penicillin which showed growth into fresh medium without antibiotic. After a 2 hr growth period in the "starting" medium, the cells were transferred at a 1-10 dilution to yield a concentration equivalent to about a 1-300 dilution of original subculture, into tubes of broth medium now containing a slightly higher series of concentrations of the penicillin. As the organisms became more resistant to 2,6-dimethoxyphenylpenicillin, growth rate decreased. The figure shows the lowest concentration of the drug which prevented growth of each successive subculture.

and DMP were equally effective. For *S. aureus*, however, a hundred-fold higher concentration of benzylpenicillin was required for half-maximum induction than of 2,6-dimethoxyphenylpenicillin.

Table IV summarizes the results on the increase in amount of penicillinase obtained with optimal concentrations of each inducer after 120 minutes induction. It is evident that DMP was a very good inducer for both *B. cereus* and *S. aureus*. However, 6-APA and benzylpenicillin were less effective inducers for *S. aureus* than they were for *B. cereus*. Weak induction systems (penicillin G with *S. aureus*; 6-APA with both *B. cereus* and *S. aureus*) were characterized not only

by lesser total amounts of enzyme produced but also by higher inducing concentrations required for induction (Table III).

TABLE III. Concentration of Inducer Producing One-Half the Maximum Amount of Induction.

Inducer	Organism	
	<i>B. cereus</i> , μg/ml	<i>S. aureus</i> , μg/ml
Penicillin G	.05	40
DMP	.05	.5
6-APA	20	2

Amounts of enzyme formed after 120 min. induction with various concentrations of inducer were plotted against concentrations. From each such plot was derived the concentration of penicillin inducer required to obtain one-half the maximum amount of enzyme.

TABLE IV. Ratio of Induced Penicillinase Activity to Basal Activity.

Inducer	Organism	
	<i>B. cereus</i>	<i>S. aureus</i>
Penicillin G	500	40
DMP	800	300
6-APA	100	20

Total penicillinase activity (per mg cell N) resulting from 120 min. induction with optimal concentrations of each inducer was compared relative to basal activity (per mg cell N) in the absence of inducer. The test organisms were logarithmically growing broth cultures. The penicillins were added when optical density reached a standard value corresponding to 10^8 cells/ml.

6-APA was a poor inducer of penicillinase in *B. cereus*, as measured by the "induction constant." However, because of its relatively low toxicity for *B. cereus* (Table II), it could be used at much higher concentrations than could the other penicillins. For *S. aureus*, 6-APA was intermediate in the concentration required to obtain half the maximum amount of enzyme, but induced at a very low rate (Table IV).

Production of mutants of S. aureus resistant to the penicillins. 6-APA, benzylpenicillin, and 2,6-dimethoxyphenylpenicillin were compared with respect to the ease with which resistant mutants could be selected by means of subculture in increasing concentrations of the antibiotics. Both a penicillinase- and a nonpenicillinase-producing strain of *S. aureus* were studied. Large inocula of organisms were used in each subculture since the mutation rate for penicillin resistance in *S. aureus* had been shown to be of the order 10^8 (10). Moderate increases in resistance to each of the penicillins was readily achieved with the non-penicillinase producing strain of *S. aureus*. Only a slight increase in resistance of the penicillinase-producing strain of *S. aureus* to 6-APA was obtained. Similarly, only a 3-fold increase in resistance to penicillin G was observed after several subcultures. However, large inocula of penicillinase-producing strain of *S. aureus* were already resistant to 10,000 μ g penicillin G per ml, a rather massive dose. In contrast, a 170-fold increase in resistance to DMP (from 2 μ g per ml to 350 μ g per ml) resulted after 6 successive subcultures (Fig. 3). The

initial subculture of the organism was fully induced with respect to penicillinase. Resistance does not appear, however, to be due to the increased amount of internal penicillinase since there was no further elevation in enzyme content as resistance increased in subsequent subcultures.

Discussion. Rolinson *et al.* (11), using whole cell preparations of *S. aureus*, have reported DMP to be stable to staphylococcal penicillinase. By employing concentrated cell-free preparations, DMP was found, in the present studies, to be hydrolyzed at measurable rates by *S. aureus* penicillinase as well as *B. cereus* penicillinase. However, DMP approaches Cephalosporin C (12) in its relative resistance to the destructive action of penicillinase.

The insensitivity of DMP to penicillinase presumably permits the intrinsic staphylocidal activity of the drug to exert itself effectively against both small and large inocula of penicillinase-producing staphylococci (13). This is in marked contrast to the failure of benzylpenicillin, at concentrations which are effective against small numbers of penicillinase-producing organisms, to inactivate larger numbers (14).

Induced formation of penicillinase in both *B. cereus* and *S. aureus* occurred with equally low concentrations of DMP. A much higher concentration of benzylpenicillin, however, was required to induce *S. aureus* than *B. cereus*. In this instance, also, the persistence of DMP against the destructive action of penicillinase, which occurs in higher concentrations in *S. aureus* than in *B. cereus*, may have contributed to its greater inducing activity.

Development of penicillin-resistant mutants of *S. aureus* appeared to proceed more readily with DMP as a selecting agent than with benzylpenicillin. Presumably the increased resistance of a penicillinase-producing strain of *S. aureus* to DMP is akin to the well-known increased resistance of nonpenicillinase-producing strains of *S. aureus* to penicillin G *in vitro*, which has yet to be adequately explained. However, the role of the penicillinase-insensitivity of DMP is dif-

difficult to assess in this situation until further study.

Summary. Benzylpenicillin, 2,6-dimethoxyphenylpenicillin, and 6-aminopenicillanic acid were compared with respect to several biochemical activities. 2,6-Dimethoxyphenylpenicillin and 6-aminopenicillanic acid were hydrolyzed at very low rates, compared to the rate for benzylpenicillin, by partially purified concentrated cell-free preparations of penicillinase derived from *B. cereus*, and at still lower rates by analogous preparations from *S. aureus*. For *S. aureus*, but not *B. cereus*, the growth-inhibitory concentrations of the two penicillinase-resistant compounds, particularly 2,6-dimethoxyphenylpenicillin, were much lower than that of benzylpenicillin. 2,6-Dimethoxyphenylpenicillin was a very effective inducer of penicillinase formation in *B. cereus* and in *S. aureus*. 6-Aminopenicillanic acid and benzylpenicillin were considerably less active as inducers of penicillinase in *S. aureus* than in *B. cereus*, as measured by effective inducing concentrations and by the amounts of enzyme formed. A mutant of a penicillinase-producing strain of *S. aureus* of increased resistance to 2,6-dimethoxyphenylpenicillin was obtained by successive subculture in increasing concentrations of the antibiotic. Its resistance did not appear to be due to the increased amount of its penicillinase content.

It is a pleasure to acknowledge the skillful assistance of Miss Emmy Lou Denny.

ADDENDUM: Examination of the manometric assay of the rate of hydrolysis of 6-APA has revealed that this method yields artifactually low results with this particular compound because the product of enzymatic hydrolysis combines with some of the CO_2 liberated from the NaHCO_3 buffer system. Iodometric assay of this reaction shows the real rates of hydrolysis to be several-fold higher than given in Table I.

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Biological Activities of 16a, 17a Dihydroxyprogesterone Derivatives. (26296)

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(Introduced by O. Wintersteiner)

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Since the demonstration by Inhoffen and Holweg that ethisterone (17a-ethinyl testosterone) is an orally active progestational agent(1), a wide variety of steroidal compounds possessing progestational activity

have been reported(2-12). Fried *et al.* have recently reported on the synthesis and progestational activity of 16a, 17a ketal derivatives of 16a, 17a-dihydroxyprogesterone (DHP), including those with acetophenone (P-DHP) and acetone (A-DHP), as well as their 6-methyl, and 6-fluoro analogues(13,

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TABLE I. Comparative Activities of 16 α , 17 α -Dihydroxyprogesterone and Certain of Its Derivatives.*

Compound	Total dose in mg											
	.125		.25		.5		1.0		2.0		5.0	
	P†	O†	P	O	P	O	P	O	P	O	P	O
Standard§	0	0	1	1	3	2	4	3	3		4	
16 α , 17 α -Dihydroxyprogesterone (DHP)							0	0			0	0
16 α , 17 α -Acetophenone of DHP	1	1	2	2	3	2	4	3	4	4	4	
16 α , 17 α -Acetonide of DHP	1		2		3	0			4	1	2	

* Numerical values of the McPhail grades are listed under the respective compounds. Each value represents mean response of at least 6 animals.

† Subcut. administration.

‡ Oral

§ Progesterone was parenteral standard and Norethisterone was oral standard.

13a). The biological activities of these derivatives of DHP are presented in this report.

Methods. New Zealand white rabbits weighing between 800 and 1200 g were used in all the progestational studies. These animals were primed with a total of 15 μ g estradiol benzoate, given in 3 divided doses on alternate days for a 6-day period, following which the steroids were administered in solution, subcutaneously or orally, daily for the next 5 days. All compounds were dissolved in sesame oil and given once daily in .2 ml doses. Animals were autopsied 24 hours following the final dose, uteri removed and weighed, and sections taken for histological evaluation according to the method of McPhail(14).

Duration of activity was determined in ovariectomized rabbits. The animals were rested for 2 weeks post operation and then estrogen-primed as described above. A single 10 mg dose of progestogen was administered intramuscularly on the day after the last priming dose was given. A maintenance dose of .5 μ g estradiol benzoate was administered every other day throughout the study. Animals were killed at 5 day intervals beginning 5 days after administration of progestogens and their uteri removed and treated as above.

P-DHP was assayed for androgenic and anti-androgenic activity according to the method of Hershberger *et al.*(15), and for uterotrophic and estrogen antagonistic properties by a modification of the method of Rubin *et al.*(16). Allen and Doisy's test

for vaginal cornification in the ovariectomized rat was used as a specific end point for estrogenicity(17). The steroid was tested for glucocorticoid activity by a modification of the rat liver glycogen deposition test of Pabst *et al.*(18), and its effect on urinary sodium and potassium was determined using a modification of the method of Kagawa *et al.*(19).

Results and discussion. The results of the progestational studies with DHP, P-DHP and A-DHP are summarized in Table I. DHP was inactive at a total dose of 25 mg when administered parenterally or orally. P-DHP and A-DHP were equal in progestational activity when parenterally administered, each being 1-2 times as potent as progesterone. However, when given orally, P-DHP was 1-2 times as active as norethisterone. (17 α -ethinyl-19 nortestosterone), the oral standard, while A-DHP was only one-eighth as active as norethisterone.

Parenteral and oral activities of the 6 β -methyl homologue of P-DHP were similar to and those of the 6 α -methyl homologue were less than those of the parent compound. No significant change in parenteral activity of A-DHP was observed following 6 α or 6 β -methylation, but both the 6 α -methyl and 6 β -methyl homologues appeared to be approximately 5 times more potent orally than A-DHP. It is interesting to note here that enhancement of progestational activity by 6-methylation parallels that observed with 6 α -methylation of 17-acetoxypregesterone (8) only in the case of the oral activity of the 6-methyl homologues of A-DHP.

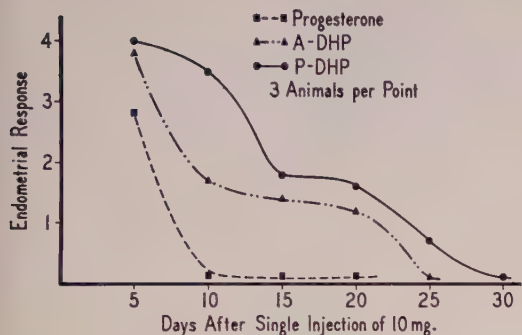


FIG. 1. Duration of progestational response following administration of progesterone, A-DHP, and P-DHP.

6 α -fluorination of the P-DHP caused a 1-2 fold increase in parenteral activity but reduced oral potency. Parenteral activity of A-DHP was unaltered by 6 α -fluorination, however, oral activity was reduced. 6 β -fluorination abolished activity by either route of administration.

Duration studies demonstrated that P-DHP and A-DHP maintain a progestational effect for 25 and 20 days, respectively, whereas a comparable dose of progesterone shows no residual luteal effect 10 days following injection (Fig. 1). P-DHP appears to act somewhat longer than A-DHP, since some activity may still be observed on the twenty-fifth day, whereas following injection of the A-DHP endometrial gland development is absent at this time.

When administered to castrated immature male rats, a 5 mg daily dose of P-DHP failed to have any effect on the weights of ventral prostate, seminal vesicles or coagulating glands, nor did it show any anti-androgenic properties when 1 mg was administered daily with 25 μ g testosterone propionate. Total doses of 100 μ g and 1 mg of this steroid exhibited moderate uterotrophic effects in the immature female mouse, and when given simultaneously with .1 μ g estradiol benzoate showed anti-estrogenic activity equivalent to that elicited by similar doses of progesterone. A 5 mg dose of the compound produced no vaginal cornification in ovariectomized rats, indicating the absence of estrogenicity. This steroid does not appear to possess glucocorticoid-like activity, as demonstrated by the

absence of glycogen deposition in the liver of the adrenalectomized rat following a 2 mg dose. When tested for electrolyte excretion, 625 μ g of P-DHP produced very slight sodium excretion and potassium retention, and when administered simultaneously with 5 μ g desoxycorticosterone acetate (DCA), no anti-DCA effect was evident.

Results of these studies demonstrate that the formation of cyclic ketals of DHP yields progestationally active derivatives from the inactive parent compound. A similar phenomenon has been previously observed with the 17 α esterification of 17 α -hydroxyprogesterone (4). However, 6 α -methylation does not enhance progestational potency in the 16 α , 17 α cyclic ketals of DHP with the same consistency as is seen with 17 α -acetoxy progesterone(8). Thus, in contrast to the additive corticoid effects of various enhancing groups introduced into the hydrocortisone molecule, such as 9 α -fluoro, Δ^1 , 16 α -methyl, 6 α -fluoro, and 16 α , 17 α cyclic ketals and acetals(11), there appears to be no general additive effect of the groups which enhance progestational activity. From the wide range of active structures reported, it would seem that a more complex relationship exists between chemical structure and progestational activity than is seen between structure and activity in the corticoid series.

Summary. The 16 α , 17 α acetophenone (P-DHP) and acetone (A-DHP) derivatives of 16 α , 17 α -dihydroxyprogesterone (DHP) and their 6 α -methyl, 6 β -methyl, 6 α -fluoro and 6 β -fluoro analogues were assayed for parenteral and oral progestational activity. P-DHP and A-DHP are equal in parenteral activity, each being about twice as potent as progesterone. P-DHP is strongly active orally, being 1 to 2 times as active as norethisterone, whereas A-DHP has only weak oral activity. Parenteral activity of P-DHP or A-DHP was not altered by 6-methylation. Oral activity of 6 β -methyl-P-DHP was similar to P-DHP but both 6 α and 6 β -methyl-A-DHP were more potent orally than A-DHP. 6 α fluorination had little or no enhancing effect on either parent compound when parenterally administered but did re-

duce oral potency. 6 β fluorination made both parent steroids inactive by either route of administration. P-DHP and A-DHP have longer duration of activity than progesterone, moderate progestational effects still being present 25 days and 20 days, respectively, following a single 10 mg intramuscular injection. P-DHP is devoid of inherent androgenic, anti-androgenic, estrogenic, anti-DCA or corticoid activities.

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Absorption of Endogenous Pyrogens by Bentonite.* (26297)

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Animals made febrile with bacterial endotoxins and myxoviruses have a substance in the circulation which is capable of producing fever when passively transferred to normal and tolerant animals(1,2). This substance which has been termed endogenous pyrogen (E.P.), is biologically distinct from the initial pyrogenic stimulus(3). E.P. has also been demonstrated in serum of animals with experimental pneumococcal(4), streptococcal(4), and staphylococcal infections(5), and in sensitized animals challenged with tuberculin(6). The E.P. found in se-

rum shares many of the biological properties of peritoneal exudates or leukocytic extracts which are also capable of producing fever (7). On this basis it has been suggested that E.P. is a product of injury to leukocytes by pyrogenic stimuli(3).

The chemical constitution of E.P. has not been determined, although recent evidence suggests that it is a protein(8). It is also not known whether endogenous pyrogens found following a variety of thermogenic stimuli are biochemically identical. One of the difficulties in answering these questions is that identification of E.P. rests solely in its ability to produce fever in animals. It seemed reasonable therefore to attempt to correlate the appearance of E.P. with the ap-

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pearance of some other product of injury to leukocytes. It has been demonstrated that lysozyme mirrors injury to leukocytes(9), and, like endogenous pyrogen, is found in increased concentration in the circulation of rabbits given bacterial endotoxins(10).

In comparing similarities between E.P. and lysozyme it was found that both substances can be absorbed from serum by bentonite and endogenous pyrogens from several sources were absorbed by this substance.

Methods. Male rabbits of mixed breed weighing 3-6 kg were housed in air-conditioned rooms and permitted rabbit pellets *ad libitum*. To record temperatures, animals were confined to metal stalls with a loosely-fitting collar. Temperatures were measured rectally by thermocouples (Telethermometer, Yellow Springs Instrument Co.).

Endogenous pyrogens were prepared by intravenous injection of 1.0 ml typhoid vaccine containing approximately 10^9 killed organisms into normal rabbits. Animals were exsanguinated 120 minutes after injection of vaccine. Blood was permitted to clot, serum cleared by centrifugation, and cultured in thioglycollate broth. If found to be sterile, sera from several animals were pooled and stored at 4°C until used. A second group of donors was given 4.0 ml of an allantoic fluid suspension of Newcastle disease virus (NDV) with hemagglutinin titer of 1:1024. Virus suspension regularly produced fever in donors. The animals were bled 4 hours later and the sera processed as described above. In a third group of animals peritoneal exudates were prepared by intraperitoneal instillation of 500 ml of 0.85% NaCl. Four hours later peritoneal fluid was aspirated by gentle suction. Most exudates contained 10^6 - 10^7 leukocytes per ml. Cells were sedimented by centrifugation and resuspended in sterile 0.85% NaCl. After mixing for 24 hours, cells were removed and cell-free saline suspensions used in experiments. These suspensions were invariably pyrogenic in normal recipient rabbits.

Bentonite was baked at 170°C for 3 hours to eliminate contamination with bacterial pyrogens. One gram of this material was thoroughly mixed with 10.0 ml of serum E.P. or

exudate and left at 4°C overnight. The bentonite was removed by centrifugation at 4°C and 5.0 ml of absorbed serum or exudate was injected into a normal recipient; the remainder was used for culture and determination of lysozyme activity. Two rabbits were used for each experiment; each received 5.0 ml of E.P. before and after absorption by bentonite. One animal was given unabsorbed E.P. on the first day and absorbed E.P. on the following day; the procedure was reversed in a second animal.

Lysozyme was determined on most aliquots of E.P. before and after contact with bentonite by the method of Kerby(9) using *Micrococcus lysodeikticus* as substrate.

In one series of experiments, dogs were given 5.0 ml of typhoid vaccine intravenously and bled 5 minutes and 120 minutes later. Sera were processed as described above and 20.0 ml aliquots were tested in normal dogs both before and after absorption with bentonite.

Precautions to avoid contamination by bacterial pyrogens of all solutions, needles and glassware were taken. Fever indices were calculated as described previously(7).

Results. Fig. 1 illustrates the mean fever curves of 6 pairs of rabbits given serum E.P. produced by administration of endotoxin or NDV, and leukocytic pyrogen derived from peritoneal exudates. Each type of E.P. produced a brisk febrile response in normal rabbits, and each contained from 6.5 to 11.0 $\mu\text{g}/\text{ml}$ of lysozyme. Following mixing of bentonite with serum or exudate, in ratio of 100 mg/ml, pyrogenic activity was almost completely eliminated and lysozyme content reduced to less than 1 $\mu\text{g}/\text{ml}$.

Reduction of pyrogenic activity was directly proportional to amount of bentonite mixed with E.P. as was demonstrated by dividing 100 ml of serum endogenous pyrogen into 5 equal parts, each of which was absorbed with 1, 10, 50, and 100 mg or no bentonite per ml E.P. respectively. 5.0 ml aliquots from each pool were tested in 3 rabbits. The results are summarized in Table I. Pyrogenic activity was almost totally abolished by absorption with 50 mg bentonite per ml E.P. On the other hand, complete

absorption of lysozyme was accomplished with 1.0 mg. In another experiment designed to assay rapidity of absorption, it was found that pyrogenicity was totally removed within 15 minutes after contact between E.P. and bentonite. Other commonly used adsorbents such as kaolin and charcoal failed to affect pyrogenic activity but absorbed lysozyme.

The effect of absorption with bentonite on the fever-producing effect of bacterial endotoxin is summarized in Table II. In this experiment the pyrogenicity of typhoid vaccine mixed with saline and serum, before and after exposure to bentonite was tested in each of 3 rabbits. Serum obtained from donor animals 5 minutes (endotoxin-in-serum) and 120 minutes (E.P.) following intravenous injection of 1.0 ml of typhoid vaccine was similarly tested. Although there was slight diminution in thermogenic activity of endotoxin-serum mixtures and endotoxin-in-serum (5 min serum) following contact with bentonite, only 120 minute-serum (E.P.) was rendered completely non-pyrogenic. Adsorp-

TABLE I. Quantitative Relationship between Amount of Bentonite Used to Absorb E.P. from Serum and Reduction in Pyrogenic and Lysozyme Activity.

Bentonite, mg/ml E.P.	% pyrogenic activity remaining	% lysozyme activity remaining
—	100	100
1.0	55	—
10	36	—
50	3.5	—
100	1.9	—

TABLE II. Fever Indices of Endotoxin, Endotoxin in Serum, and Serum E.P. before and after Absorption by Bentonite.

Material tested	Fever index	
	Un-absorbed	Absorbed
1.0 ml TV + 4.0 ml N. saline	22.8	30.0
1.0 " " + 4.0 " NRS	42.5	30.9
5 ml 5' serum	20.8	13.6
5 " 120' "	15.8	3.0

tion to bentonite also failed to affect the pyrogenicity of NDV.

In another experiment, E.P. obtained from dogs following administration of typhoid vaccine was tested in these animals before and after admixture with bentonite. The results were similar in every respect to those obtained in rabbits and will not be detailed.

Discussion. The observation that several biologic characteristics of lysozyme and E.P., such as reduction of pyrogenic and lyso-genic activity by bentonite, tend to parallel one another suggests that lysozyme may be a useful, easily-measured indicator of E.P. which does not entail use of animals. Parallel measurements of both substances need to be carried out under a variety of conditions to determine more precisely the relationship between lysozyme and E.P. Since lysozyme has been found to be an index of injury to leukocytes, the present findings support the idea expressed by Atkins and Wood (1), that E.P. appears when granulocytes are damaged by a variety of pyrogenic stimuli.

A number of silicate minerals under proper conditions of pH and solvent composition are known to adsorb charged particles. Bentonite has predominantly exposed negative charges at certain points and hence positively charged particles are adsorbed to it(11).

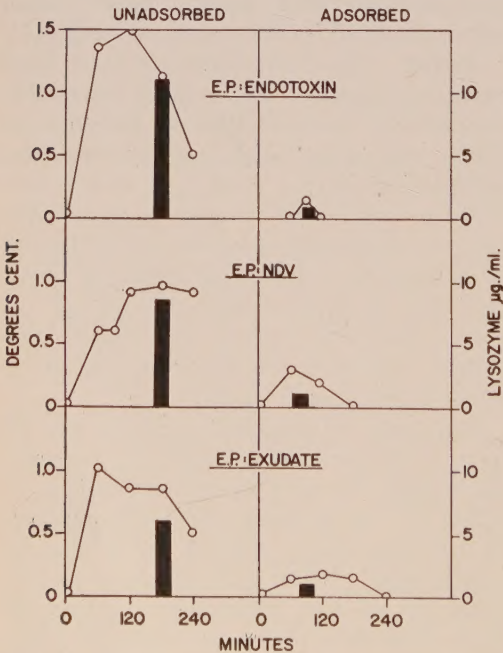


FIG. 1. Pyrogenic activity of endogenous pyrogens produced by administration of endotoxin and NDV, or in leukocytic exudates before and after absorption with bentonite. Lysozyme activity decreased concomitantly.

The probability that E.P. carries a net positive charge is consistent with its failure to be adsorbed to DEAE, an anionic exchange cellulose(8). The ability of bentonite to inactivate endogenous pyrogens produced by a variety of methods suggests that these fever-producing substances carry the same charge. However, the precise chemical structure of several types of E.P. remains to be determined. It is also of interest that the pyrogenic activity of typhoid vaccine was not altered by bentonite, adding further support to the idea that endogenous pyrogen is different from exogenously administered pyrogens. On the other hand, serum-endotoxin mixtures were inactivated to a limited extent, perhaps because the augmenting effect of normal serum on endotoxin was partially reduced(12).

The mechanism governing the action of bentonite upon E.P. is not clear. It is most probable that the silicate merely absorbs E.P. from serum or leukocytic extracts but attempts to elute E.P. from bentonite have met only with sporadic success. In the majority of instances eluates from bentonite have been non-pyrogenic. These negative results may be due to failure to break the bond between E.P. and bentonite, inactivation of E.P. during adsorption or elution, or elution of only part of the E.P. absorbed. The concentration of the E.P. in these partially eluted samples was probably too small to elicit a pyrogenic response. Experiments are in progress

to increase the yield of these eluates.

Summary. Endogenous pyrogen (E.P.) in serum from animals given endotoxin or Newcastle disease virus and in leukocytic exudates was inactivated by absorption with bentonite. Concentration of lysozyme in serum and exudates paralleled that of E.P. The pyrogenicity of typhoid vaccine and NDV was not affected by absorption with bentonite.

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